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Detection of a new race of *Meloidogyne enterolobii* in cotton crop, genetic diversity of races, and resistance in *Gossypium* spp.

Detecção de uma nova raça de *Meloidogyne enterolobii* na cultura do algodoeiro, diversidade genética de raças e resistência em *Gossypium* spp.

CAIO FELIPE DE BARROS SOUZA

BRASÍLIA - DISTRITO FEDERAL
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Detecção de uma nova raça de *Meloidogyne enterolobii* na cultura do algodoeiro, diversidade genética de raças e resistência em *Gossypium* spp.

Thesis presented to the University of Brasília as a partial requirement for obtaining the title of Doctor in Phytopathology from the Graduate Program in Phytopathology.

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SUMMARY

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General Abstract: Detection of a new race of *Meloidogyne enterolobii* in cotton crop, genetic diversity of races, and resistance in *Gossypium* spp.

Meloidogyne incognita is a well-known root-knot nematode (RKN) species that infects cotton globally. Recently, new resistant cultivars to *M. incognita* were released in Brazil, being considered the best control strategy. *Meloidogyine enterolobii*, not historically considered a major threat to cotton production, has caught the attention due to recent reports in the United States and Brazil, causing severe damage in *M. incognita* cotton resistant cultivars, highlighting its potential as an epidemic RKN. In 2019, the first infection by *M. enterolobii* on resistant cotton (IMA 5801B2RF) was reported in Minas Gerais state, Brazil. Subsequently, in 2021 *M. enterolobii* was detected again in the municipality of São Desidério, western Bahia state, on the same resistant cotton cultivar. Another survey in cotton fields cultivated with resistant 'IMA 5801B2RF' from six municipalities in Bahia state (three different geographical origins) were identified by esterase phenotypes (EST) and SCAR markers as *M. incognita*, but *M. enterolobii* was not found again, confirming its possible restricted occurrence in western Bahia state. A bioassay with the resistant cotton cultivar in greenhouse conditions demonstrated robust reproduction of *M. enterolobii* (RF=12.8), but no reproduction of the field populations of *M. incognita* (FR<1.0), indicating the lack of virulence of these populations to the resistant cotton. The concatenated neighbor-joining tree showing the genetic variability analysis grouped the *M. enterolobii* race 1 populations (guava, pepper and sweet potato) and the two Brazilian cotton populations (race 2) separately, and with high bootstrap support (100%). The sweet potato population showed the greatest divergence from the other populations. Mitochondrial (COII), ribosomal DNA (ITS, D2-D3), and HSP90 gene studies revealed limited interactions related to geographical origin or races of *M. enterolobii*. The North Carolina differential host test (NCDHT) identified two

physiological races: race 1 (from guava, pepper and sweet-potato) and race 2 (from cotton), with distinct pathogenic profiles. We evaluated the performance of current Brazilian cultivars as alternatives for *Meloidogyne* spp. race tests, and tomato 'Santa Clara', pepper 'Magali R', watermelon 'Crimson Sweet', peanut 'IAC Tatu', tobacco 'NC4', and cotton 'FM966' can be recommended as a substitute for old cultivars suggested in NCDHT. Genetic resistance is the most promising approach for managing root-knot nematodes. We tested twenty-four cotton accessions, aimed to identify sources of resistance to *M. enterolobii* in Embrapa's cotton germplasm including different *Gossypium* species and hybrids under greenhouse conditions. Artificial inoculations were performed, and after 120 days, various variables including gall index, egg mass index, total number of eggs per gram of roots, and reproduction factor were assessed. While some genotypes showed susceptibility, others, particularly Upland genotypes and hybrids, exhibited varying levels of resistance. Notably, genotype CNPA GO 2002-2043/5 consistently demonstrated partial resistance. Despite the virulence of *M. enterolobii*, certain cotton genotypes with known resistance QTLs showed significant reductions in nematode populations after inoculation, highlighting the potential of selecting resistant cotton genotypes as a viable strategy to mitigate nematode impact on cotton crops.

Keywords: Guava root-knot nematode, physiological races, RAPD, AFLP, ITS, D2D3, ITS, COII, esterase phenotypes, SCAR markers, resistance.

Resumo Geral: Detecção de uma nova raça de *Meloidogyne enterolobii* na cultura do algodoeiro, diversidade genética de raças e resistência em *Gossypium* spp.

Meloidogyne incognita é a espécie de nematoide das galhas mais importante para o algodoeiro no Brasil e em todas as área produtoras dessa *commoditie* no mundo. Recentemente, a cultivar resistente IMA 5801B2RF e outras similares foram lançadas, no Brasil, visando ao controle deste nematoide. *Meloidogyne enterolobii*, embora historicamente não fosse considerado uma grande ameaça para a produção de algodão, chamou a atenção devido aos relatos recentes, no Brasil e nos Estados Unidos, de danos severos em cultivares de algodoeiro resistentes a *M. incognita*, destacando seu potencial como praga da cultura. Em 2019, foi relatada a primeira infecção por *M. enterolobii* em algodoeiro resistente no estado de Minas Gerais, Brasil. Posteriormente, em 2021, *M. enterolobii* foi detectado no município de São Desidério, no oeste do estado da Bahia, na mesma cultivar de algodoeiro resistente. Em continuidade, um estudo investigou áreas de algodoeiro com a cultivar resistente 'IMA 5801B2RF' em seis municípios do estado da Bahia. As seis populações de três origens geográficas diferentes foram identificadas por fenótipos de esterases (EST) e marcadores SCAR como *M. incognita. Meloidogyne enterolobii* não foi detectado em nenhuma dessas amostras, dando indícios de ocorrência restrita no oeste da Bahia. Em um bioensaio com o algodoeiro 'IMA 5801B2RF' resistente, em condições de casa de vegetação, observou-se boa reprodução de *M. enterolobii* (RF=12,8), mas não das populações de campo de *M. incognita* (FR<1,0), indicando a não virulência dessas populações para o algodoeiro resistente. Posteriormente, a variabilidade genética de sete populações de *M. enterolobii* de diferentes origens geográficas e raças foram avaliadas com o uso de 44 primers RAPD e 7 AFLP. A análise agrupou as populações da raça 1 de *M.enterolobii* (goiabeira, pimentão

e batata doce) e as duas populações brasileiras do algodoeiro (raça 2) separadamente e com alto suporte de *bootstrap* (100%). A população de batata-doce foi a mais divergente em relação às outras populações. Estudos das regiões mitocondriais (*COII*), do DNA ribossômico (*ITS*, *D2-D3*) e do gene *HSP90* revelaram interações limitadas relacionadas à origem geográfica ou raças de *M. enterolobii*. O Teste de hospedeiros diferenciais da Carolina do Norte (NCDHT) identificou duas raças fisiológicas: raça 1 (provenientes da goiaba, pimentão e batata-doce) e raça 2 (provenientes do algodão), com dois perfis patogênicos distintos só diferenciados pelo algodoeiro. Avaliou-se também a eficácia de cultivares brasileiras atuais como alternativas para testes de raça de *Meloidogyne* spp., e o tomateiro 'Santa Clara', o pimentão 'Magali R', a melancieira 'Crimson Sweet', o amendoim 'IAC Tatu', o tabaco 'NC4' e o algodoeiro 'FM966' podem ser recomendados como substitutas para as antigas cultivares sugeridas no NCDHT. A resistência genética é vista como uma abordagem promissora para o manejo de nematoides das galhas. Testaram-se vinte e quatro acessos de algodoeiro, com o objetivo de identificar fontes de resistência a *M. enterolobii* no germoplasma de algodoeiro da Embrapa, incluindo diferentes espécies de *Gossypium* e híbridos, em condições de casa de vegetação. Inoculações artificiais foram realizadas e, após 120 dias, os índices de galhas e de massas de ovos, o número total de ovos por grama de raiz e o fator de reprodução foram avaliados. Alguns genótipos mostraram suscetibilidade, mas outros, incluindo diversos híbridos de algodoeiro, exibiram diferentes níveis de resistência. Notavelmente, o genótipo CNPA GO 2002-2043/5 foi resistente a *M. enterolobii*. Apesar da agressividade de *M. enterolobii*, alguns genótipos de algodoeiro com QTLs de resistência já mapeados mostraram redução significativa na população final de nematoides, destacando o potencial de seleção de genótipos resistentes a outros nematoides como uma estratégia

viável para mitigar o impacto de novas espécies de nematoides nas lavouras comerciais da cultura.

Palavras chave: nematoide da goiabeira, raças fisiológicas, RAPD, AFLP, fenótipos de esterase, marcadores SCAR, ITS, D2D3, COII.

General Introduction

Cotton (*Gossypium* spp.) is an economically important crop that primarily provides natural fibers for the textile industry and oil derived from the seeds, for food and biofuel production. Among the most recurrent nematodes in cotton cultivation, the root-knot nematode, *Meloidogyne incognita* (Kofoid and White, 1919) Chitwood, 1949, is considered the most significant due to its wide range of hosts and its ability to cause economic losses through direct or indirect damage to the plant's root system (Ogallo *et al.*, 1997).

One of the most reliable control strategies for *Meloidogyne* spp. is genetic resistance, due to its economic advantages combined with effectiveness. Currently, there are two main sources of resistance to *M. incognita* available for cotton, one derived from *Gossypium hirsutum* L. and the other from *G. barbadense* L. (Lopes *et al.*, 2020). In Brazil, many cotton cultivars with these *G. hirsutum* resistance genes have been recently introduced, such as IMA 5801B2RF, FM 912GLTP, FM 970GLTP, BRS 500B2RF and BRS 800B3RF. These cultivars have also been developed using transgenic technologies, incorporating resistance to Lepidopterous pests and herbicides (Belot *et al*., 2020; Suassuna *et al*., 2021). In the United States, quite a few cotton cultivars with resistance genes to *M. incognita* are available from four different seed companies with these same genes from Upland cotton (Wheeler *et al*., 2020).

Recently, the species *M. enterolobii* Yang and Eisenback, 1983 was detected in Brazil parasitizing cotton by Galbieri *et al*. (2020). The species was originally described in 1983 from a population causing severe damage to the pacara earpod tree (*Enterolobium contortisiliquum* (Vell.) Morong) in China (Long *et al.*, 2014). In 1988, a new species of the genus, named *M. mayaguensis* (Rammah and Hirschmann, 1988), was described from a population in Puerto Rico, which was later synonymized as *M. enterolobii* based on esterase phenotype, mitochondrial DNA sequence and morphological approaches (Hunt and Handoo, 2009; Karssen *et al*., 2012). To date, this nematode exhibits a wide host range, parasitizing over 67 species of cultivated plants across 27 botanical families (Castillo and Castagnone-Sereno, 2020). There is a concern regarding *M. enterolobii* due to its ability to develop in genotypes of host crops carrying resistance genes to the root-knot nematodes, such as in pepper (*Tabasco* and *N genes*), tomato (*Mi-1* gene), soybean (*Mir1* gene), potato (*Mh* gene), cowpea (*Rk* gene), and sweet potato (Koutsovoulos *et al.*, 2020b).

Since the description of these species, *M. enterolobii* and *M. mayaguensis* have been classified as belonging to distinct host cycles. The first one parasitizes all differentiating hosts except peanut (equivalent to race 4 of *M. incognita*) (Yang and Eisenback, 1983), and the second one parasitizes the same hosts except peanut and cotton (equivalent to race 2 of *M. incognita*) (Rammah and Hirschmann, 1988). In Brazil and worldwide, there are few studies on *M. enterolobii* races, but there are reports indicating that populations affecting guava trees throughout the Brazilian territory belong to the race equivalent to *M. incognita* race 2, which does not parasitize cotton (Moura and Moura, 1989; Carneiro *et al.*, 2006; Carneiro *et al*., 2021). However, Galbieri *et al*. (2020) detected this same species parasitizing cotton (equivalent to race 4 of *M. incognita*) in the state of Minas Gerais, Brazil.

A study of intraspecific variability using three different genetic markers (AFLP, ISSR, and RAPD) demonstrated low variability and genetic distance among 16 populations of *M. enterolobii* collected from different hosts and geographic locations (Tigano *et al.*, 2010). Sequencing of the mitochondrial gene *COI* (*mtCOI*) from 19 populations of *M. enterolobii* collected in China also revealed low intraspecific variability and no correlation between genetic and geographic distance (Shao *et al.*, 2020). However, in any of these previously mentioned studies the concept of other races of *M. enterolobii* were included.

Currently, few sources of resistance to *M. enterolobii* are known, to date the *Ma* gene from Myrobalan plum (*Prunus cerasifera* Ehrh.) (Claverie *et al.*, 2011), in guava (*Psidium* spp.) (Freitas *et al.*, 2014), and some pepper accessions (*Capsicum* spp.) (Gonçalves *et al.*, 2014). Recently, Embrapa released the rootstock for guava 'BRS Guaraçá' with resistance to *M. enterolobii*, which is being marketed in the main guava-producing regions of Brazil (Carneiro *et al*., 2021). Other sources of resistance to *M. enterolobii*, such as accessions of *Gossypium* spp., including *G. barbadense* and *G. arboreum*, available in the Embrapa's germplasm bank were not studied. In addition to the search for new sources of resistance, efforts are needed to use different screening methods, considering cotton as semi-perennial crop.

Therefore, it is of great interest to detect and study different populations and races of *M. enterolobii* on different crops like soybean (Verssiani *et al*., 2023), guava (Sousa *et al*., 2024), vegetables like tomato and pepper not yet studied, and the resistant cotton cultivar IMA 5801B2RF, exploring the genetic variability of different races and biotypes of *M. enterolobii*.

Objectives

General

To identify virulent populations of *Meloidogyne* spp. parasitizing the resistant cotton IMA 5801B2RF in western Bahia state; determine races of *M. enterolobii* detected in different crops in Brazil using different host varieties and genetic diversity of these populations; screening new sources of resistance in *Gossypium* spp. to *M. enterolobii* cotton race.

Specifics

• To identify populations of *Meloidogyne* spp. in the cultivar IMA 5801B2RF, in root samples collected from cotton fields in the state of Bahia using esterase (Est) phenotypes and SCAR markers and test the virulence of these populations on cotton plants in green-house conditions.

• To determine the races of *M. enterolobii* populations from different crops, using classical host differentials test methodology and providing new set of differential hosts plants using current crop varieties.

• To study the genetic variability of *M. enterolobii* races from cotton and other crops using AFLP, RAPD markers, and sequencing of the *ITS*, *D2-D3*, *HSP90* and *COII* gene regions.

• To select new sources of resistance on cotton to *M. enterolobii* in Embrapa's *Gossypium* spp. accessions.

CHAPTER 1 – LITERATURE REVIEW

1. Cotton trade and cultivation

The cotton is an oilseed plant belonging to the Malvaceae family, within the genus *Gossypium*, encompassing approximately 50 species. Among them, only four hold economic importance. *Gossypium hirsutum* L., constituting around 90% of the global fiber production, is predominant in the Brazilian cultivation system (Galbieri *et al.*, 2009). *G. barbadense* L. is the second most cultivated species, accounting for approximately 5% of the world's fiber production, while *G. arboreum* L. and *G. herbaceum* L. contribute to the remaining 5% of cotton production (Bolek, 2016).

Global cotton production reached approximately 27 million tons in 2023, with India as the largest producer, followed by the United States, China, and Brazil. Brazilian production reached 3.04 million tons in 2023, being 47% destined for export (USDA, 2023). Among the Brazilian states producing cotton, Mato Grosso leads with over 961,000 hectares planted and a production of 1.8 million tons of cotton lint in the 2021/2022 season, followed by the state of Bahia with 266 thousand hectares planted and a production of 527 thousand tons (CONAB, 2022).

Cotton is a tropical plant, requiring long, hot, and humid summers for optimal vegetative development. Most current cotton cultivars last five to seven months to complete their vegetative cycle (Freire, 2007). On the other hand, the maturation period requires dry weather, as rainfall during this phase can negatively impact fibre quality and yield (Carvalho and Ferreira, 2006). The significant expansion of cotton cultivation areas in Brazil, linked with substantial investment in research, has led to the adoption of new technologies in major producing regions (Central-West and Northeast). For cotton, new varieties have provided a more defined growth cycle, high productivity, increased lint

yield, pest resistance, herbicide tolerance, and resistance to major diseases (Galbieri *et al.*, 2018).

2. The genus *Meloidogyne*

2.1. Meloidogyne *on cotton*

Root-knot nematodes belong to the genus *Meloidogyne* Göldi, 1887, a genus with over 100 described species. Among them, *M. incognita*, *M. javanica* (Treub) Chitwood, 1949, and *M. arenaria* (Neal, 1889) Chitwood, 1949 are polyphagous species commonly found in tropical and subtropical regions (Carneiro et al., 1996; Devran and Baysal, 2018). Two species of root-knot nematode are considered parasites of cotton: *M. incognita* and *M. acronea* Coetzee, 1956. *Meloidogyne incognita* is globally significant, causing substantial damage to global cotton crops (Starr *et al*., 2005), while *M. acronea* is restricted to southern Mali and semi-arid regions of South Africa (Bridge, 1992; Starr *et al*., 2005).

Damage to cotton caused by *M. incognita* varies according to population density in the field. Losses of 20 to 35% in cotton lint yield per hectare were detected in crops in southern Mato Grosso when the population in 5g of root exceeded 100 specimens (Silva *et al.*, 2014; Belot and Galbieri, 2016). The root-knot nematode has four physiological races, being races 3 and 4 pathogenic to cotton (Hartman and Sasser, 1985), and race 3 being the most common in cotton fields in Brazil (Belot and Galbieri, 2016; Perina *et al.*, 2017).

Recently, the species *M. enterolobii* (= *M. mayaguensis*) was detected for the first time in Brazil parasitizing cotton (Galbieri *et al*., 2020). Since the description of the species, *M. enterolobii* and *M. mayaguensis* have been classified as belonging to two different hosts patterns. *Meloidogyne enterolobii* parasitizes all differentiating hosts (tomato, pepper, watermelon, tobacco, cotton) except for peanut (equivalent to race 4 of

M. incognita) (Yang and Eisenback, 1983), and *M. mayaguensis* parasitizes the same hosts except for peanut and cotton (equivalent to race 2 of *M. incognita*) (Rammah and Hirschmann, 1988). In Brazil and worldwide, there are few studies on races of *M. enterolobii*, but there are reports showing that populations from guava distributed throughout the national territory (Carneiro *et al*., 2021) belong to the race equivalent to race 2 of *M. incognita*, which does not parasitize cotton (Moura and Moura, 1989; Carneiro *et al.*, 2006). However, Galbieri *et al*. (2020) detected this *M. enterolobii* parasitizing cotton (equivalent to race 4 of *M. incognita*) in the state of Minas Gerais. The life cycle of the root-knot nematode (Figure 1) involves the stages of egg inside gelatinous matrix, four juvenile stages (J1-J4), and the adult phase, predominantly females with occasional male formation. The first ecdysis occurs inside the egg, where the nematode transitions from the J1 stage to J2. The J2 stage hatches from the eggs through the mechanical force of the stylet and the release of chitinases produced by the nematode.

The J2 (second-stage juveniles) are the mobile and infective form that penetrate the roots through the elongation region and migrate towards the subapical meristem, returning through the central region of the root to the maturation region in the vascular cylinder. This is the site of establishment of feeding cells, known as giant cells. The feeding sites consist of four to eight multinucleated cells with dense cytoplasm, induced by the nematode through control of the host plant's mitotic cell cycle (Sikandar *et al.*, 2023).

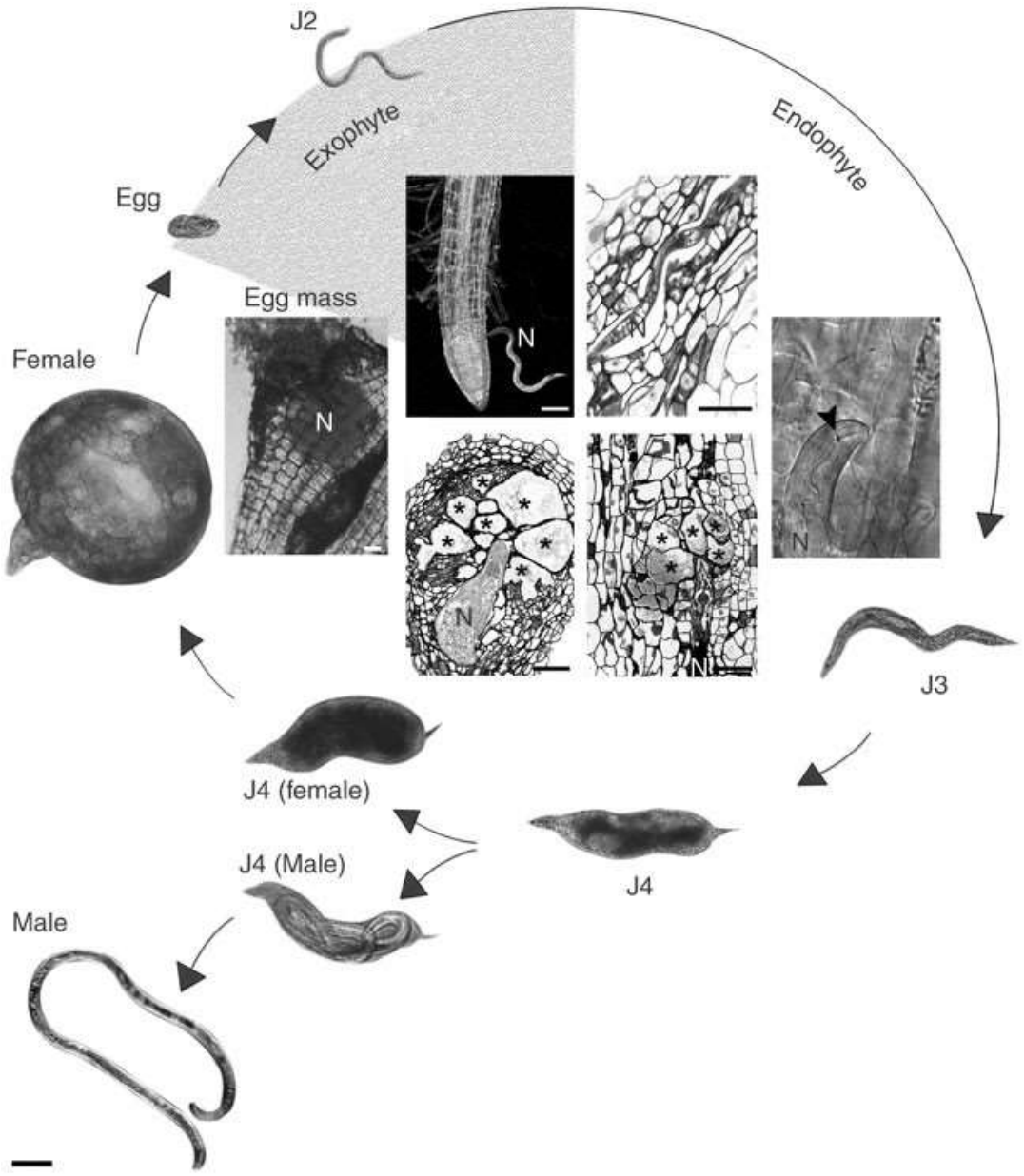

Figure 1 - Life cycle of *Meloidogyne* spp. Image: Abad *et al*. (2008). Available at < https://doi.org/10.1038/nbt.1482>

The control of the plant cell cycle involves the accumulation of polyploid nuclei in each cell (karyokinesis) without cytokinesis, i.e., division of the cytoplasm to form a new cell. The cells adjacent to the feeding cells undergo hyperplasia and hypertrophy due to the host's excessive production of auxins and other hormones, leading to the formation

of galls on the roots. The giant cells function as a large biological sink, deflecting the upward and downward flow of water and nutrients from the conducting vessels, xylem, and phloem, respectively, enriching the nematode's feeding sites (Belot and Galbieri, 2016). The nematode life cycle takes 28 to 37 days and is negatively influenced by environmental factors such as soil temperatures (below 18°C or above 42°C) and soil moisture (below 21%) (Goodell and Ferris, 1989; Chen *et al*., 2009). Under favourable conditions, J2 forms within 14 days, while the combined J3 and J4 stages take 4-6 days to form (Ferraz and Monteiro, 2011).

The adult forms have a complete reproductive system, with pear-shaped females and thread-like males. Females exhibit high fertility, producing and releasing up to 500 eggs in a gelatinous matrix. The combination of the gelatinous matrix with the eggs is called an egg mass, serving as protection against soil desiccation and soil microorganisms (Ferraz and Monteiro, 2011).

The damage caused in susceptible plants involves direct and reflex symptoms. Direct symptoms can be observed in the roots by the presence of galls and underdeveloped root systems. Reflex symptoms are seen in the aboveground part as underdevelopment, mineral deficiency, water deficiency associated with wilting, and leaf curling (Koenning *et al.*, 2004; Belot and Galbieri, 2016).

2.2. Meloidogyne enterolobii *and guava decay*

Meloidogyne enterolobii was originally described from a population causing severe damage to the pacara earpod tree (*Enterolobium contortisiliquum* (Vell.) Morong) on Hainan Island, China. Based on perineal patterns of females, it was preliminarily identified as *M. incognita*. However, through a morphological approach, the population was found to be significantly different of *M. incognita*, leading to the description of a new root-knot nematode species named *M. enterolobii* (Xu *et al.*, 2004). A few years later,

another root-knot nematode species was described from specimens recovered from eggplant roots with severe gall symptoms in Puerto Rico and named *M. mayaguensis*. In its original description, the authors indicated that this species resembled *M. enterolobii* but differed from it in some morphological characteristics (Rammah and Hirschmann, 1988).

More recently, the taxonomic relationship between these two species has been further questioned based on molecular data. Xu *et al*. (2004) demonstrated the similarity of sequences from a mitochondrial DNA (*mtDNA*) region between these two species, suggesting that *M. mayaguensis* should be considered a synonym of *M. enterolobii* (Castagnone-Sereno, 2012). The official synonymy was established by Karssen *et al*. (2012) through a comparison of the holotypes and paratypes of these two species, using morphological and morphometric approaches.

Root-knot nematode (*Meloidogyne* spp.) is considered a limiting factor for guava's commercial production in Central and South America and other countries (Carneiro *et al*., 2021). In Cuba, Puerto Rico, Mexico, Venezuela, and Brazil, guava production has decreased in the last 30 years and is attributed to the increasing of *Meloidogyne* spp. in the crops (El-Borai and Duncan, 2005). The only species of the genus *Meloidogyne* that occurs in guava is *M. enterolobii* (Carneiro *et al*., 2021), recognized as the guava nematode.

Many detections made in the past, using the perineal region as an identification tool, inaccurately showed the presence of *M. incognita*, *M. javanica*, and *M. arenaria* instead of *M. enterolobii* (Carneiro *et al*., 2021). Indeed, for this species of nematode, the perineal region showed very different perineal profiles (Carneiro *et al*., 2001; Brito *et al*., 2004), leading to confusion with other species. More recently, research conducted in Costa Rica, Vietnam, India, and South Africa using enzymatic and molecular markers

showed that the only species occurring in guava is *M. enterolobii* (Carneiro and Almeida, 2001).

In the São Francisco Valley, a major guava producing region in northeast Brazil, 70% reduction was reported in guava production within a seven year period. Direct losses associated with guava infection by *M. enterolobii* in several states in Brazil might reach up to US$ 61 million, not to mention direct job losses as a result of decline in guava orchards. (Freitas *et al.,* 2014)

The spread of *M. enterolobii* to various Brazilian states occurred through the trade of guava seedlings infected by the nematode (Carneiro *et al*., 2021). However, its natural occurrence in other locations cannot be disregarded. For example, in the state of Paraná, the occurrence of this nematode in guava trees was reported without apparent introduction of seedlings from other locations (Lima *et al*., 2005; Carneiro *et al*., 2006).

2.3. Biochemical identification of nematodes of the genus Meloidogyne

The use of isoenzymatic markers, such as esterase and malate dehydrogenase profiles, allowed an accurate identification of various *Meloidogyne* species and demonstrated the reliability of these techniques (Blok and Powers, 2009; Carneiro, 2016; Monteiro *et al.*, 2019, Carneiro *et al*., 2024).

The first conclusive study of the identification of *Meloidogyne* species, using individualized females through esterases phenotype, was conducted by (Janati *et al.*, 1982), who studied 86 populations and identified 75 of them at the specific level. Subsequently, Esbenshade and Triantaphyllou (1985), in collaboration with the International *Meloidogyne* Project (IMP), studied about 300 populations from 65 countries and various continents. They reported esterase patterns of various *Meloidogyne* species, with the most common phenotypes being A2 and A3 (*M. arenaria*), H1 (*M. hapla*

Chitwood, 1949), I1 (*M. incognita*), and J3 (*M. javanica*). Some cryptic species were characterized in other works and later described or identified (Carneiro *et al*. 1996; 2000, 2016, 2024). The authors found 21 esterase phenotypes typical of 21 *Meloidogyne* species from Brazil, characterizing the cryptic species using Integrative Taxonomy (Carneiro *et al*., 2016, 2024).

In a didactic manner (Figure 2), Carneiro *et al*. (2024) provides a diagram where enzymatic phenotypes are designated using the first letter as the species name and the number of bands of that species in the polyacrylamide gel (Example: *M. javanica*, Est J3). Species with the same initials and number of bands are distinguished by small letters (Example: *M. enterolobii*, Est En2, *M. hispanica* Hirschmann, 1986, Est Hi3). Enzyme patterns are generally compared with a known standard of *M. javanica* (Est J3), which should be included in electrophoresis gels to determine the relative migration distances (Rm.).

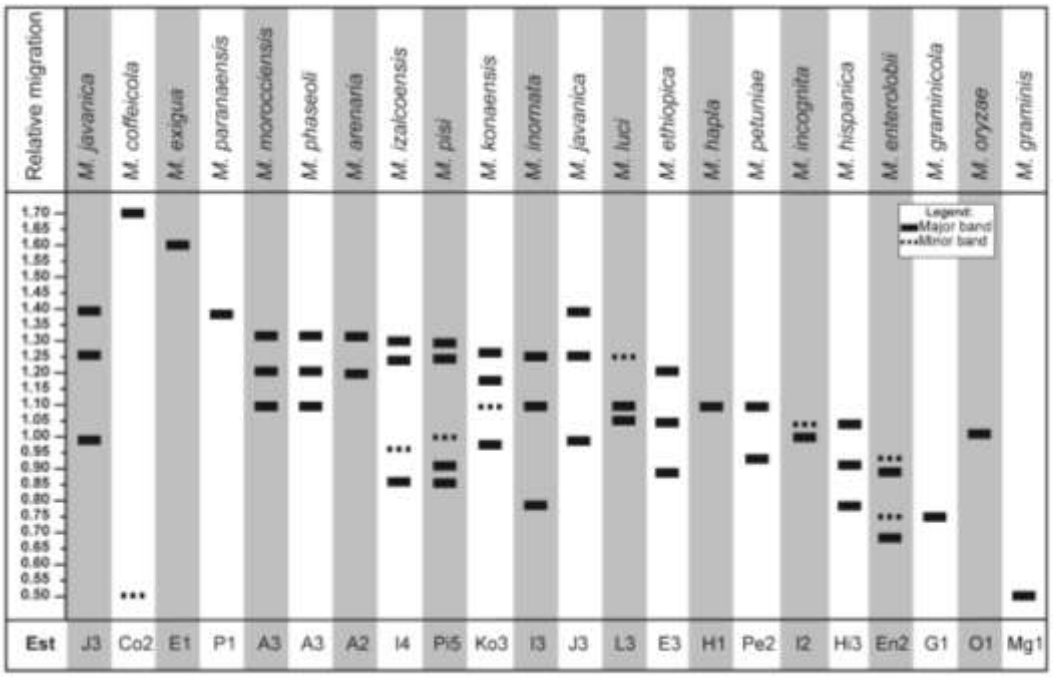

Figure 2 - Esterase (Est) phenotypes of 21 *Meloidogyne* species detected in Brazil. Relative migration (Rm): migration ratio in relation to the slowest band of *M.*

javanica (Rm=1.0). Image: (Carneiro *et al*., 2024). Available at < https://doi.org/10.1007/978-1-0716-3638-1_5>

The main advantages of this technique are the specific detection of *Meloidogyne* spp., even in mixed populations, identification of atypical or cryptic populations, efficiency, reliability, and quickness (Carneiro and Almeida, 2001; Blok and Powers, 2009; Carneiro *et al*., 2017). So far, there are no enzymatic patterns for all the nearly 100 described species in the genus *Meloidogyne*. One disadvantage is that these isoenzymatic markers cannot be used in studies of intra-specific variability, which require reasonable levels of variability (Correa *et al.*, 2013). Intra-specific variability at the enzymatic level is generally very low. Enzymes produced through the expression of highly conserved genes represent only a small fraction of the functional genome, while non-coding regions are more abundant and exposed to evolutionary changes at a higher frequency (McLain *et al*., 1987).

2.4. Molecular identification of nematodes in the genus Meloidogyne

Alternatively, to isoenzyme detection, DNA-based detection tools constitute an excellent method for diagnosing species in the genus *Meloidogyne*. These methods are independent of phenotypic variation and interpretation, simple, precise, fast, and can be used in high-throughput approaches (Castagnone-Sereno *et al.,* 2011; Carneiro *et al.,* 2017).

Polymerase Chain Reaction (PCR) identification methods are primarily based on target DNA regions of nematodes using species-specific primers. Primers for the diagnosis of *Meloidogyne* species have been developed based on conserved regions in DNA (*ITS* 1 and 2; *IGS* 1 and 2 and *ETS*) (Perry *et al.,* 2007).

The current approach involves converting Random Amplified Polymorphic DNA (RAPD) markers into Sequence Characterized Amplified Region (SCAR), allowing the

composition of longer, GC-rich, and sequence-specific primers. SCAR-PCR is highly sensitive and enables the detection of species even in mixed populations at proportions equal to or lower than 1% (Randig *et al*., 2002). Among other advantages, it includes the use of physical reference points in the genome, serving for mapping or as species-specific genetic markers when associated with some genotype or phenotype of interest (Mienie *et al.*, 2002).

In root-knot nematodes, SCAR markers have been developed to identify two quarantine species, namely *M. chitwoodi* Golden, O'Bannon, Santo & Finley, 1980, and *M. fallax* Karssen, 1996. Additionally, SCAR markers have been designed to distinguish the three species, *M. incognita*, *M. javanica*, and *M. arenaria*, primarily found in tropical and subtropical regions or in protected cultivation systems (Zijlstra *et al*., 2000; Meng *et al*., 2004). Species-specific SCAR markers have been defined for the three main *Meloidogyne* species in coffee plants*: M. exigua* Göldi, 1887, *M. incognita*, and *M. paranaensis* Carneiro, Carneiro, Abrantes, Santos, & Almeida, 1996 (Randig *et al*., 2002). This set of highly sensitive primers can identify three different species in a single reaction. Recently, primers have also been developed for the species *M. enterolobii* (Tigano *et al.*, 2010) and *M. izalcoensis* Carneiro, Almeida, Gomes & Hernandez, 2005 (Correa *et al.*, 2013).

Although molecular techniques for identifying nematodes of the genus *Meloidogyne* have been developed and disseminated, they are still not capable of identifying all species within this genus. However, they can currently identify approximately 18 species. For this reason, it is recommended to use a diagnostic approach for root-knot nematodes based on more than one technique, including biochemical and morphological approaches.

2.5. Reaction of Differential host test

The term "race" for the genus *Meloidogyne* does not have the same meaning as physiological race used in other groups of plant pathogens. By definition, races are biotypes differentiated by their host preference within a taxonomic group. In this case, the hosts are cultivars of a plant species, unlike the usual separation of races of *Meloidogyne* spp. that involves plants from different species and even botanical families (Moura, 1996).

Species of *Meloidogyne* naturally exhibit different feeding preferences; however, when these differences occur within the same species, they are referred to as physiological races (Eisenback and Triantaphyllou, 1991). In *Meloidogyne* spp., parasitic races are differentiated through positive and negative reactions observed in plants of different species, such as tomato (*Solanum lycopersicum* L. 'Rutgers'), tobacco (*Nicotiana tabacum* L. 'NC 95'), cotton (*Gossypium hirsutum* 'Deltapine 61'), bell pepper (*Capsicum annuum* L. 'Early California Wonder'), watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai 'Charleston Gray'), and peanut (*Arachis hypogaea* L. 'Florunner'), based on the differential host test from North Carolina State University (Hartman and Sasser, 1985) (Table 1).

The variation in the range of differential host plants for certain *Meloidogyne* species has been known since 1954 when Joseph Neal Sasser developed a simple method based on the response of a series of differential host plants to identify the four main species of *Meloidogyne* recognized by Chitwood in 1949 (*M. incognita*, *M. hapla*, *M. arenaria*, and *M. javanica*) (Eisenback and Triantaphyllou, 1991). This test has also been used as a crucial component in describing new species, proposing the demonstration of a single host reaction and continues to be utilized in recent species descriptions.

Since 1970s, with a significant increase in the number of described species, the responses of some new species to differential hosts overlapped with the races already determined for the four main species, for example in *M. paranaensis*, which exhibits a reaction similar to *M. javanica* race 1, or *M. ethiopica* Whitehead, 1968 with the same reaction as *M. incognita* race 2, or *M. inornata* Lordello, 1956, which has a reaction equivalent to *M. arenaria* race 2. *Meloidogyne paranaensis* was identified for many years as *M. incognita* race 5 or 'biotype IAPAR', due to the perineal pattern of *M. incognita* and host reactions similar to *M. javanica* (Carneiro *et al.,* 2016).

Table 1 - The North Carolina differential host race test for sixteen root-knot nematode species (Adapted from Hartman and Sasser, 1985)

Meloidogyne **species and races**	**Tobacco 'NC 95'**	**Cotton 'Deltapine 61'**	**Pepper 'California Wonder'**	**Watermelon 'Crimson Sweet'**	**Peanut 'Florunner'**	**Tomato 'Rutgers'**
M. incognita						
Race 1	-	-	+	+	-	+
Race 2	+	-	+	+	-	+
Race 3	-	+	+	+	-	+
Race 4	+	+	+	+	-	+
M. javanica						
Race 1	+	-	-	+	-	+
Race 2	+	-	+	+	-	+
Race 3	+	-	-	+	+	+
Race 4	+	-	+	+	+	+
M. arenaria						
Race 1	+	-	+	+	+	+
Race 2	+	-	-	+	-	+
M. exigua						
Race 1	-	-	+	-	-	+
Race 2	-	-	+	-	-	-
Race 3	-	-	-	-	-	-
M. hapla	+	-	+	-	-	+
M. paranaensis	+	-	-	+	-	+
M. enterolobii	+	-	+	+	-	+
M. ethiopica	+	-	-	+	-	+
M. inornata	+	-	-	+	-	+
M. morocciensis	+	-	+	+	-	+
M. petuniae	+	-	-	-	-	+
M. izalcoensis	+	-	+	+	-	+
M. phaseoli	+	-	-	-	-	+

M. pisi	+	-	-	-	-	+
M. luci	+	-	+	-	-	+
M. konaensis	+	-	-	+	-	+

An extensive study involving the genome sequencing of 11 populations of *M. incognita* belonging to different physiological races was conducted, but no correlation between phylogeny and races was observed. No genetic determinism was found with epigenetic factors being suggested (Koutsovoulos *et al.*, 2020b). However, a high genetic and even morphological diversity between *M. arenaria* race 1 and race 2 was reported by Carneiro *et al*. (2008).

While races have been recognized in the four main species of *Meloidogyne*, Moens *et al*. (2009) recommend discontinuing the use of the terminology. The term "race" has never been universally accepted since this concept measures only a small variation among populations of the same species, whose host range is extensive in most species of root-knot nematodes. However, knowledge of races is essential for characterizing resistance in genetic improvement programs and for management measures, particularly in implementing crop rotation strategies (Fassuliotis, 1985; Lordello and Lordello, 1996; Subbotin *et al.*, 2021).

2.6. Genetic Diversity of Meloidogyne spp.

The development of molecular techniques allowed new perspectives and opened for studies on intraspecific variability of root-knot nematodes. The advancement of PCR techniques has brought significant progress in the implementation of new molecular markers.

With the development of DNA amplification techniques using small primer and random sequences, the use of PCR became widespread worldwide. This allowed the genetic analysis of various species at relatively low costs and in a more simplified manner,

as it did not require the high costs associated with DNA sequencing or the high risk associated with the use of radioactive probes (Caetano-Anollés *et al*., 1991; Hadrys *et al*., 1992). Several studies have been conducted based on the analysis of markers such as RFLP (Restriction Fragment Length Polymorphism), RAPD (Random Amplified Polymorphisms DNA), and ISSR (Inter-Simple Sequence Repeats) with promising results for the genetics of *Meloidogyne* populations (Castagnone-Sereno *et al.*, 1993; Randig *et al.*, 2009; Tigano *et al.*, 2010).

More recently, with the development of new sequencing tools and consequently the reduction in sequencing costs, new approaches for variability analysis and phylogenetic relationships have become available. Regions of ribosomal DNA (rDNA) (e.g., 18S, ITS, the D2-D3 segment of the 28S subunit, and the intergenic spacer [IGS-2]), mitochondrial DNA (mtDNA) (cytochrome oxidase 1: COI, COII/16S, NADH5), have received significant interest (Blok, 2005; Trinh *et al.*, 2022).

2.7. Molecular markers: RAPD and AFLP

The RAPD technique, based on PCR, is currently used in genetic studies and for differentiating *Meloidogyne* species, based on profiles generated with random primers (Petersen *et al.*, 1997; Randig *et al.*, 2002). It relies on small amounts of genetic material and does not require prior knowledge of the genome being studied (Williams *et al.*, 1990). The main limitation of RAPD markers is the low genetic information content per locus. Only one allele is detected, while other allelic variations are collectively classified as a null allele (presence or absence). RAPD markers, therefore, behave as dominant markers, and the data have a binary nature (Ferreira and Grattapaglia, 1996).

The PCR-AFLP technique relies on amplifying a subset of fragments generated from genomic DNA digestion with combinations of type II restriction enzymes, which cleave DNA at specific sites (4 – 8 bp; usually in palindromic sites). The technique is

based on the property of certain restriction enzymes to leave cohesive ends (sticky ends) of known sequences after DNA cleavage. Thus, it is possible to construct double-stranded nucleotide sequences that bind to the ends of these restriction fragments. Once the adapter sequence and the restriction site are known, specific primers can be constructed for pre-amplification of the restriction fragments (Ferreira and Grattapaglia, 1996).

The first study on the diversity of Brazilian populations of *Meloidogyne* spp. was conducted by Randig *et al*. (2002) using PCR-RAPD, analysing 18 populations of *Meloidogyne* spp. The results revealed that *Meloidogyne* species differentiated into groups according to the enzymatic profiles described for each species. Additionally, the study showed a high degree of intraspecific variability in populations of *M. exigua*, *M. hapla*, and *M. arenaria*, with a polymorphic fragment rate exceeding 50% of total generated fragments. In contrast, populations of *M. incognita* and *M. javanica* exhibited low intraspecific variability, less than 30%.

Species with parthenogenetic reproduction typically exhibit low intraspecific genetic variability. Parthenogenesis facilitates rapid reproduction since mating is unnecessary, unlike in amphimictic species. However, plant-parasitic nematode species characterized by parthenogenetic reproduction, such as *M. javanica*, *M. arenaria*, and *M. incognita*, demonstrate genetic variation that enables swift adaptation to unfavourable environments, such as soils cultivated with resistant host plants (Castagnone-Sereno, 2006).

2.8. Molecular markers: D2D3, ITS and COII

The ribosomal DNA (*rDNA*) remains one of the most comprehensive tools for phylogenetic studies. Ribosomal DNA sequences are responsible for synthesizing ribosomal RNAs (*rRNAs*), essential components in cellular physiology for protein synthesis. In eukaryotic organisms, the main ribosomal locus consists of three regions:

18S rDNA, *5.8S rDNA*, and *28S rDNA*. These three genes are transcribed into a single RNA, separated by two regions: the internal transcribed spacers (*ITS*), *ITS-1* between the *18S* and *5.8S* genes, and *ITS-2* between the *5.8S* and *28S* genes (Hillis and Dixon, 1991).

Ribosomal genes have many desirable characteristics of an evolutionary marker, which is why they have been used to analyse relationships across a wide range of taxa and levels of divergence. However, they also have some undesirable characteristics that can affect phylogenetic analyses and must be considered. These include variability in evolutionary rate among taxa and among genes, structural and functional limitations that result in differences in substitution frequency among gene regions, the occurrence of multiple mutations in the same region, which can obscure phylogenetic signal, and the process of genetic recombination during mitosis (Hillis *et al*., 1996).

For the study of *ITS* regions, it is possible to design PCR primers based on the most conserved genes of the *28S*, *5.8S*, and *18S* loci, which flank the *ITS* regions. The sequences amplified by these primers can be useful for phylogenetic comparisons between distant taxa. However, *ITS* regions are particularly useful for distinguishing between very closely related taxa, as they evolve more rapidly than coding regions (Hillis and Dixon, 1991).

Mitochondrial DNA (*mtDNA*) has been extensively used for molecular and phylogenetic studies of nematodes in the *Meloidogyne* genus (Powers *et al*., 1986; Hyman, 1988; Hugall *et al.*, 1994; Stanton *et al*., 1997). Its high copy number and faster evolutionary rate compared to nuclear genomes make it particularly valuable for such analyses (Moritz *et al.*, 1987).

The *mtDNA* region has been reported to be highly variable in length, producing amplification products that differ in repetitive unit length for *M. incognita*, *M. javanica*,

and *M. arenaria* (Stanton et al., 1997). It has been suggested that this region could be useful for distinguishing different species and physiological races of *Meloidogyne* spp. (Hugall *et al*., 1994; Stanton *et al*., 1997). However, subsequent studies have shown no correlation between the genome and physiological race, limiting its use as a molecular marker for intraspecific characteristics of *Meloidogyne* spp. (Koutsovoulos *et al.*, 2020b). In contrast to the high variability found in this region for *M. incognita*, *M. javanica*, and *M. arenaria*, Blok *et al*. (2002) showed that for *M. enterolobii*, this region is short with fewer variations.

3. Root Knot Nematode Management Strategies

Plant-parasitic nematodes are found in nearly all agricultural crops worldwide, reducing both production and crop quality and causing significant economic losses. The most effective method for controlling plant pathogens in general is exclusion, aiming to prevent the pathogen entering in the area. Nematodes can be easily spread by human activities that create a "bridge" between contaminated and healthy areas, such as the transportation of soil infested with machinery and tools, plant debris, contaminated seedlings, and irrigation water (Collange *et al.*, 2011). A notable example of nematode dissemination through human activity was the commercialization of guava seedlings infected with *M. enterolobii*, originating from Petrolina, Pernambuco (Carneiro *et al*., 2021).

The use of nematicides in global agriculture has been increasingly discouraged worldwide due to the persistence of chemical compounds in the soil, contamination of groundwater, and harmful effects on humans and the environment. Other negative factors associated with the use of nematicides include rising production costs and their temporary effectiveness (Ferreira, 2014). Additionally, nematicides show low efficiency in terms of

efficacy against new nematode biotypes and the potential for generating populations tolerant to the active ingredient (Chitwood, 2003).

Biological control relies on the antagonistic relationship between microorganisms and nematodes, characterized by various modes of action such as competition for space and nutrients, antibiosis, parasitism, growth promotion, and induction of host plant resistance (Sikora 1992; Dong and Zhang, 2006; Tian *et al*. 2007; Carneiro *et al*., 2021). For *Meloidogyne* control, several biological agents have been studied, and some are already recommended, including bacteria like *Pasteuria penetrans* (Thorne) Sayre and Starr, *Bacillus megaterium* Barry, and *B. subtilis* (Ehrenberg) Cohn, as well as trap-forming fungi like *Arthrobotrys* spp. and *Monacrosporium* spp., or egg-parasitic fungi such as *Pochonia chlamydosporia* (Goddard) Zare and W. Gams, *Purpureocillium lilacinum* (Thom) Luangsa-ard, Houbraken, Hywel-Jones, and Samson, and *Trichoderma* spp. (Nordbring-Hertz *et al*., 2001; Tranier *et al*. 2014; Silva, 2015; Carneiro *et al*., 2021; Timper *et al*., 2021).

Cultural management methods of plant-parasitic nematodes are those in which agricultural practices are modified to control the density of these parasites or reduce their negative impact on crops. These measures can be applied in soil preparation, fertilization, sowing, weed management, harvesting, crop residue elimination, succession, and crop rotation, among others (Collange *et al.*, 2011; Inomoto, 2016). Crop rotations are an effective method of improving yields.

Research suggests that effective control of soybean cyst nematode (SCN) can be achieved by alternating SCN-resistant soybean cultivars with corn (Howard *et al*., 1998; Chen *et al.*, 2001; Long and Todd, 2001). Rotating to a non-host crop for at least one year can help reduce damage caused by root-knot nematodes (RKN) and reniform nematodes (Davis *et al.*, 2003; Hauer *et al.*, 2016). However, nematode levels may recover to pre-

rotation population levels after a season of growing a susceptible crop. For this reason, the use of genetic resistance is aimed for an effective control.

Genetic control involves the use of resistant cultivars, depending highly on the availability in the market of cultivars or rootstocks that combine resistance to nematodes with high productivity (Starr *et al*. 2007; Carneiro *et al*., 2021;).

Currently, new sources of genetic resistance to root-knot nematodes have been identified. In plants with resistance genes, the development of the ideal feeding site for the nematode is not observed since necrosis and death of cells, often lead to the degradation of giant cells (hypersensitivity reaction, HR). Consequently, nematodes fail to establish suitable feeding sites and die due to starvation (Milligan *et al.*, 1998; Lopes *et al.*, 2020). The use of genetic resistance against plant-parasitic nematodes is highly recommended, if available, as it does not increase production costs and is not harmful to non-target microorganisms.

4. Resistance for RKN on cotton

Cotton (*Gossypium* spp.) is the most important natural fibre crop worldwide. The diversity of *Gossypium* species also provides an ideal model for investigating the evolution and domestication of polyploids. However, the cotton's vast and complex genome poses challenges to genomic research. Technical advances in high-throughput sequencing and bioinformatic analysis have largely overcome these obstacles, leading in a new era of cotton genomics (Chen *et al.*, 2007; Shao *et al.*, 2020).

The use of resistant cultivars is the most accessible method for nematode control, as it doesn't increase production costs or cause environmental imbalance (Davis and Stetina, 2016). Currently, known sources of resistance to root-knot nematodes in cotton include genotypes ‘Auburn 634RNR’ (derived from crossing ‘Auburn 623RNR’ with

‘Auburn 56’), ‘TX 25’ (*G. hirsutum* race punctatum), ‘CIR 1343’, and ‘CIR 1348’ (*G. barbadense*), as well as ‘M 315RNR’ (from crossing Auburn ‘634RNR’ with ‘Deltapine 16’) and ‘Acala Nem X’, with an unknown origin (Silva *et al*., 2014). Additionally, the cultivar Fai Mui (*G. arboreum*) has shown resistance (Mota *et al.*, 2013).

Although, resistance in *G. barbadense* and *G. arboreum* has been demonstrated through studies on their reaction to *M. incognita* (Mota *et al.*, 2013; Lopes *et al.*, 2020), molecular markers are currently only available for two QTLs derived from *G. hirsutum*. These markers include SSR BNL 3661 (for the chromosome 14 QTL), which inhibits the formation or proper functioning of the feeding cells, reducing the quantity of eggs produced by the *M. incognita* females, and CIR 316 (for the chromosome 11 QTL), which acts early, hindering the development of J2 stage to subsequent nematode stages (Jenkins *et al*., 2012; Gomez *et al*., 2016; Alves *et al*., 2017; Lopes *et al*. 2020).

Currently, the United States seed market has several cotton cultivars released with resistance genes to *M. incognita* from four seed companies (Wheeler *et al*., 2020). Recently in Brazil the commercial cultivars IMA 5801B2RF, FM 970GLTP RM, FM 912GLTP RM, BRS 500 B2RF and BRS 800B3RF were released carrying the same resistance genes from M 315 (Belot *et al*., 2020; Suassuna *et al*., 2021). The source of resistance genes presents in commercial cultivars in the United States and Brazil originated from Auburn 623 RNR, a result of a cross of two moderately resistant genotypes (Clevewilt 6 and Wild Mexican Jack Jones), providing oligogenic resistance from genes located on chromosomes 11 (*qMi-C11*) and 14 (*qMi-C14*) (Shen *et al.*, 2006; Ynturi *et al.*, 2006).

Several studies highlighted the high resistance of M 315 RNR lines (Shepherd *et al.*, 1996; McPherson *et al.*, 2004; Lopes *et al.*, 2020) and M-240 RNR (Shepherd, 1983; Starr and Smith, 1999) to *M. incognita*, and no virulent populations have been found

against genotypes possessing the *c11* and *c14* genes, such as IMA 5801 B2RF. However, virulent populations of *M. incognita* against cotton cultivars carrying at least one of these resistance genes are known, such as the Acala Nem X and Stoneville LA 887 cultivars (Zhou *et al*., 2000). LA 887 carries the same chromosome 14 QTL as M-315 RNR (Gutiérrez *et al.*, 2010). Mechanisms leading to the overcoming of resistance genes in plants by *M. incognita* nematodes are attributed to the loss of copies of convergent genes aimed at adapting to resistance genes (Castagnone-Sereno *et al.*, 2019). Thus, despite the proven stability of the high resistance conferred by the *c11* and *c14* genes, there is a possibility of their overcoming by virulent populations of the root-knot nematode.

Until now, sources of resistance to *M. enterolobii* have not been studied in *Gossypium* spp. accessions, and resistance sources for this species are only known in *Prunus cerasifera*, *Psidium* spp., and *Capsicum* spp. (Claverie *et al.*, 2011; Freitas *et al.*, 2014; Gonçalves *et al.*, 2014).

CHAPTER 2 - OCCURRENCE OF A NEW RACE OF *Meloidogyne enterolobii* AND AVIRULENT *M. incognita* POPULATIONS PARASITIZING COTTON IN WESTERN BAHIA STATE, BRAZIL[1]

Abstract - *Meloidogyne incognita* is the root-knot nematode (RKN) species globally known to infect cotton plants, and the resistant cultivar IMA 5801B2RF has recently been released in Brazil for its control. In 2019, the first infection by *M. enterolobii* on resistant cotton was reported in Minas Gerais state, Brazil. In 2021, in our previous survey, this nematode species was detected again in Brazil in the municipality of São Desidério, western Bahia state on the same resistant cotton cultivar. In a continuous study, we have surveyed cotton fields from six municipalities in Bahia state, in areas supposedly cultivated with the resistant cotton; all the six populations from three different geographical origins were *M. incognita*, but *M. enterolobii* was not found again, confirming its restricted occurrence in western Bahia state. The RKN identifications were based on biochemical and molecular approaches and revealed the phenotype α-esterase Est En2 for *M. enterolobii* and Est I2 for *M. incognita* and typical SCAR patterns of these species, confirming the enzymatic identification. In soil and root analysis we detected second-stage juveniles (J2) of *M. incognita* in 21 samples (50% of occurrence) and *Rotylenchulus reniformis* in 13 samples (31%) extracted from soil and roots and identified using taxonomic approaches. A bioassay with the resistant cotton cultivar in greenhouse conditions revealed a high reproduction of *M. enterolobii* (RF = 12.8) but did not allow

[1] Results published in scientific journal: Souza CFB, Galbieri R, Belot J-L, Negri BF, Perina FJ, Cares JE, Carneiro RMDG (2022). Occurrence of a new race of *Meloidogyne enterolobii* and avirulent *M. incognita* populations parasitizing cotton in western Bahia state, Brazil. Physiol Mol Plant Pathol 121:101874. https://doi.org/10.1016/j.pmpp.2022.101874

reproduction of the field populations of *M. incognita* (RF < 1.0), showing that these populations of *M. incognita* were not virulent to this resistant cotton. There may have been an incorrect identification in the field cultivar and/or seed mixture in on-farm seed production. Our study provided the first report of a natural infection of a new race of *M. enterolobii* on cotton and highlighted the absence of virulent *M. incognita* populations on cotton in western Bahia state, Brazil.

Keywords: Root-knot nematode. Detection. *Gossypium hirsutum*. Races.

Resumo - OCORRÊNCIA DE UMA NOVA RAÇA DE *Meloidogyne enterolobii* E POPULAÇÕES AVIRULENTAS DE *M. incognita* PARASITANDO ALGODÃO NO OESTE DO ESTADO DA BAHIA, BRASIL

Meloidogyne incognita é globalmente reconhecida por infectar plantas de algodão, e a cultivar resistente IMA 5801B2RF foi recentemente lançada no Brasil para o seu controle. Em 2019, foi o primeiro relato da ocorrência natural de *M. enterolobii* parasitando algodoeiro resistente no estado de Minas Gerais, Brasil. Em 2021, em um levantamento prévio, essa espécie de nematoide foi detectada novamente no Brasil no município de São Desidério, no oeste do estado da Bahia, na mesma cultivar de algodão resistente. Em sequência foi realizado um levantamento em diferentes áreas de produção algodão de seis municípios no estado da Bahia, em áreas supostamente cultivadas com o algodão resistente; todas as seis populações de três origens geográficas diferentes foram identificadas como *M. incognita*, mas *M. enterolobii* não foi encontrada novamente, confirmando sua ocorrência restrita no oeste da Bahia. As identificações dos nematoides das galhas foram baseadas em abordagens bioquímicas e moleculares e revelaram o fenótipo α-esterase Est En2 para *M. enterolobii* e Est I2 para *M. incognita*, e padrões

SCAR típicos dessas espécies, confirmando a identificação enzimática. Na análise do solo e das raízes, detectamos juvenis de segundo estágio (J2) de *M. incognita* em 21 amostras (50% de ocorrência) e *Rotylenchulus reniformis* em 13 amostras (31%), extraídos do solo e das raízes e identificados usando abordagens taxonômicas. Um bioensaio com a cultivar de algodão resistente em condições de estufa revelou uma boa reprodução de *M. enterolobii* (RF = 12,8), mas não ocorreu a reprodução das populações de campo de *M. incognita* (RF < 1,0), mostrando que essas populações de *M. incognita* não se mostraram virulentas para o algodão resistente. Esses resultados sugerem uma identificação incorreta da cultivar em campo e/ou mistura de sementes na produção *on farm*. Este estudo foi o primeiro relato da infecção natural de uma nova raça de *M. enterolobii* em algodão e destacou a ausência de populações virulentas de *M. incognita* em algodão resistente no Oeste do estado da Bahia, Brasil.

Palavras chaves: Nematoide das Galhas. Detecção. *Gossypium hirsutum*. Raças.

1. Introduction

Cotton (*Gossypium hirsutum* L.) is a crop of great importance for the world trade balance, especially for Brazil, the second world's largest exporter of this commodity (USDA, 2021). However, biotic agents such as nematodes can cause damage to this crop, considerably reducing its productive potential (Robinson, 2008). So far, only three root-knot nematode (RKN) species of the genus *Meloidogyne* Göldi, 1887 have cotton parasite status. These are *M. incognita* (Kofoid and White, 1919) Chitwood, 1949 (races 3 and 4), which is considered the most important cotton nematode due to its widespread occurrence in crop areas and its potential damage (Starr *et al*., 2005); *M. acronea* Coetzee, 1956 isolated in southern Africa; and, more recently, *M. enterolobii* Yang and Eisenback, 1983, which was detected on cotton in the USA (Ye *et al.*, 2013) and in Brazil (Galbieri *et al.*, 2020).

Meloidogyne enterolobii was originally described from a population that caused severe damage on the pacara earpod tree in China (Yang and Eisenback, 1983). A few years later, a new species of root-knot nematode was described from specimens from galled roots of eggplant in Puerto Rico, and named *M. mayaguensis* (Rammah and Hirschmann, 1988). In 2004, Xu *et al.* (2004) demonstrated sequence identity of an mtDNA region between these two species and suggested that *M. mayaguensis* should be considered as a junior synonym of *M. enterolobii*. In addition, the esterase phenotype of *M. mayaguensis* from Puerto Rico is identical to *M. enterolobii* from China (Esbenshade and Triantaphyllou, 1985). A possible confusion and misidentification of *M. mayaguensis* as *M. enterolobii* has also been signaled by different authors and reported in detail by Carneiro *et al*. (2021). The official synonymization was finally established by Karssen *et al.* (2012) by comparing the holo and paratypes of these two species, using morphological and morphometrical approaches.

Meloidogyne enterolobii was initially thought to be restricted to tropical areas, but recent reports show its detection in the Mediterranean region and other subtropical areas, resulting in the designation of *M. enterolobii* as a quarantine pathogen by several countries and regulatory agencies (Elling, 2013). This is due to its exceptionally wide host range that includes many vegetables, guava, acerola, ornamentals, soybean, weeds and cotton; in addition, it is due to its capacity to overcome resistance genes in several crops (Castagnone-Sereno, 2012; Elling, 2013; Galbieri *et al.*, 2020).

In Brazil, *M. enterolobii* was only found causing severe damage and wide dispersion in different states in the guava crop, due to the planting of contaminated seedlings from the Northeastern region, where there are many guava nurseries (Carneiro *et al*., 2021). In other crops, sporadic or limited occurrences were recorded. In previous

studies, the race of *M. enterolobii* that parasitizes guava did not parasitize cotton (Carneiro *et al.*, 2006).

In the USA (Ye *et al.*, 2013), *M. enterolobii* has only been detected in the Goldsboro region**,** Wayne County, North Carolina. The first report of *M. enterolobii* parasitizing cotton in Brazil occurred in 2019 in the state of Minas Gerais (Galbieri *et al.*, 2020), in plants carrying two resistance genes to *M. incognita*, *qMi-C11* and *qMi-C14* (Belot *et al.*, 2020). The plants showed reduced size and root galls, larger than those induced by *M. incognita* infections (Galbieri *et al.*, 2020).

One of the most reliable control strategies for *Meloidogyne* spp. is genetic resistance, due to economic aspects and its effectiveness, as was reported for guava rootstock 'BRS Guaraçá' (Carneiro *et al.,* 2021). Currently, the United States seed market has several cotton cultivars released with resistance genes to *M. incognita* from four seed companies (Wheeler *et al.*, 2020). Recently in Brazil, the commercial cultivar IMA 5801B2RF was released by Instituto Mato-Grossense do Algodão (IMA), carrying the same resistance genes. The source of resistance genes presents in commercial cultivars in the United States and Brazil originated from Auburn 623 RNR, a result of a cross of two moderately resistant genotypes (Clevewilt 6 and Wild Mexican Jack Jones), providing oligogenic resistance from genes located on chromosomes 11 (*qMi-C11*) and 14 (*qMi-C14*) (Shen *et al.*, 2006; Ynturi *et al.*, 2006). Until now, no population of *M. incognita* has been able to overcome the oligogenic resistance conferred by the two resistance QTLs.

In this context, the main objectives of the present study were: (i) to identify species of *Meloidogyne* and other nematodes parasitizing a resistant cotton cultivar carrying *c11* and *c14* genes in the western region of Bahia, Brazil; (ii) to determine the percentage of occurrence of *Meloidogyne* spp. and other nematodes in the same region to calculate the distribution of *M. enterolobii* populations associated with resistant cotton roots; and (iii)

to confirm or deny the virulent nature (resistance breakdown) of *M. incognita* populations, through bioassays with resistant and susceptible cultivars under greenhouse conditions.

2. Materials and Methods

2.1. Nematode sampling and reproduction

Sampling was carried out in the municipalities of Barreiras, Correntina, Baianópolis, Jaborandi, Riachão das Neves and São Desidério, located in the state of Bahia, in areas supposedly planted with cultivar IMA 5801B2RF, totalling 12,600 hectares (ha) of sampled area. Areas with the resistant cultivar and presenting symptoms caused by *Meloidogyne* spp. infection (Figures 3, 4) were identified based on information provided by the farmers. Ten subsamples (soil and roots) were collected at a depth of 0-20 cm. A fraction of 200 cm^3 of soil was processed by the method described by Jenkins (Jenkins, 1964). The roots were separated, washed and 10 g was processed by the method of Coolen and D'Herde (1972). The identification and quantification of soil and root specimens were performed using Peter's glass slide under light microscope for the genus (Mai, 1988) and for species level in the case of *Rotylenchulus* (Jatala, 1991). Egg extraction for RKN multiplication used the modified method of Hussey and Barker (Hussey and Barker, 1973) (0.5% sodium hypochlorite solution); the eggs were extracted from cotton roots and were inoculated in tomato (*Solanum lycopersicum* L., cv. Santa Clara) plants and maintained at 25–30 °C under greenhouse conditions for 120 days.

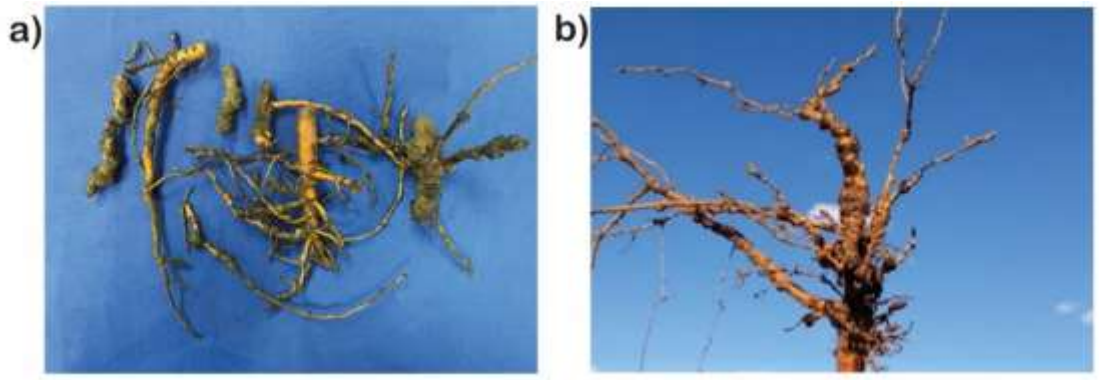

Figure 3 - Roots of cotton plants parasitized by *Meloidogyne enterolobii* showing large galls. a) roots from Minas Gerais state, b) roots from Bahia state. Photos: Carneiro, R.M.D.G. (a) and Galbieri, R. (b)

2.2. Biochemical identification of Meloidogyne species

Females from infected plants were extracted individually from cotton or tomato plants and characterized biochemically by the electrophoresis technique, using the enzyme α-esterase (Est), in polyacrylamide gel, according to the methodology described by Carneiro and Almeida (2001). Confirmation of inoculum purity was carried out with this technique. The obtained isozyme profiles were compared and confirmed according to the ones described in the literature (Carneiro *et al.*, 2016).

2.3. Identification of the Meloidogyne species by SCAR markers

The extraction of 200-400 µl of eggs from each population was done according to Carneiro *et al.* (2004). Total genomic DNA was extracted according to the method described by Randig *et al*. (2002), quantified and then stored at -20°C. The identification was done using the SCAR species-specific primers: MK7-F (5'-GATCAGAGGCGGGCGCATTGCGA-3') and MK7-R (5'-CGAACTCGCTCGAACTCGAC-3') for *M. enterolobii* (Tigano *et al.*, 2010)*;* for *M. incognita* the primers Inck14-F (5'- GGGATGTGTAAATGCTCCTG-3') and Inck14-R (5'- CCCGCTACACCCTCAACTTC-3') (Randig *et al.*, 2002).

2.4. Reproduction of Meloidogyne enterolobii and M. incognita populations on resistant and susceptible cultivars

To confirm the virulence of *M. enterolobii* and *M. incognita* populations from western Bahia region, a bioassay was carried out in two cotton genotypes, IMA 5801B2RF (resistant) and TMG 44B2RF (susceptible). The population of *M. enterolobii* from Bahia and six populations of *M. incognita* from three municipalities were selected for the bioassay due to the high number of juveniles (>500) of *M. incognita* found in soil and cotton roots: Riachão das Neves (Sample ID: BA-211142), São Desidério (Pool, sample IDs: BA-210824, BA-210825 and BA-210826) and Correntina (Pool, sample IDs: BA-211132 and BA-211133). Four plants of each cotton genotype were grown in pots (20×15 cm) filled with a mixture of autoclaved soil and Bioplant® compost (1:1) and maintained at 25–30 °C, under greenhouse conditions. Twenty-five days after seedling emergence, pots were inoculated with 5,000 eggs of *M. enterolobii* or *M. incognita*, extracted according to the modified methodology of Hussey and Barker (Hussey and Barker, 1973), in which the roots were ground in a blender with 0.5% sodium hypochlorite (NaOCl) solution for 30 seconds. Plants were arranged in a randomized block design and watered and fertilized as needed. Four months after inoculation, the root systems were rinsed under tap water and weighed; eggs were extracted by the same technique, using NaOCl 1%. The reproduction factor (RF) was calculated as RF = FP/IP, where FP = final population and IP = initial population (5,000 eggs). The average RF was transformed as log (x+1), submitted to analysis of variance and the means grouped using the Scott-Knot test ($P<0.05$).

3. Results

3.1. Nematode sampling and quantification

In June 2021, in a previous survey, in a cotton field located in the state of Bahia, in the municipality of São Desidério (12°21'37"S/44°58'48"W), plants of the cultivar IMA 5801B2RF were found showing reduced height and a low number of bolls. The roots showed large galls similar to the symptoms described by Galbieri *et al*. (2020) (Fig. 4a, b). The plants were located in an irrigated field with a total area of 130 ha, with coarse-loamy soil (15-20% clay) where cotton had been planted for 20 years. The extraction of eggs from 450 g of roots, from field samples, resulted in a population of 19,074.38 eggs/g of roots, a high population of *Meloidogyne* sp. on resistant cotton. The number of second-stage juveniles in soil/roots was also evaluated (Table 2).

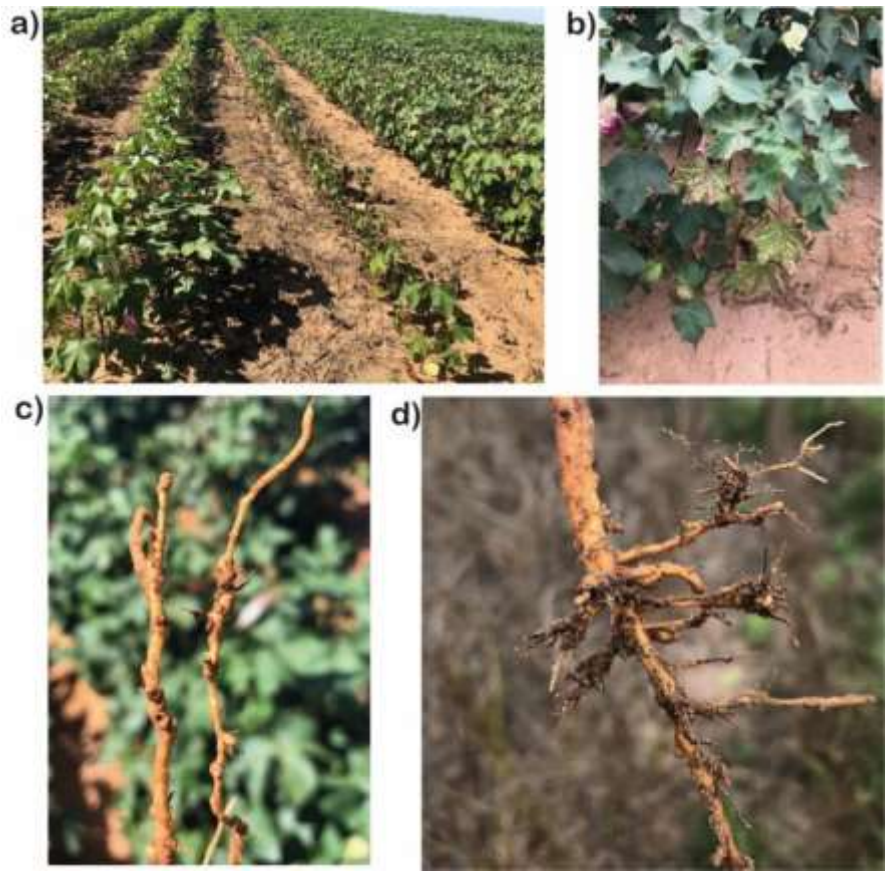

Figure 4 - Above-ground and root symptoms caused by *Meloidogyne incognita* on cotton plants from sampled area in Bahia state, Brazil. a: stunting of plants; b:

yellowing between leaf veins (interveinal chlorosis); c and d: small, irregular root galls. Photos: Souza, C. F. B.

Table 2 - Number of second-stage juveniles (J2) found in cotton plants collected in western Bahia.

Sample ID	Geographic Origin	Coordinates	J2 of *Meloidogyne* spp. per 200cm³ of soil + 10g of roots	J2 of *Rotylenchulus reniformis* per 200cm³ of soil + 10g of roots
BA-210800 [ab]	São Desidério	12°46'53"S/45°57'058"W	2525	0
BA-210401	Barreiras	11°47'52"S/45°39'087"W	19	0
BA-210402	Barreiras	11°47'52"S/45°39'087"W	12	0
BA-210403	Barreiras	11°47'44"S/45°39'046"W	0	44
BA-210404	Barreiras	11°48'41"S/45°39'281"W	0	0
BA-210405	Barreiras	11°46'09"S/45°39'005"W	0	0
BA-210406	Riachão das Neves	11°50'48"S/45°74'763"W	0	17
BA-210407	Riachão das Neves	11°50'48"S/45°74'763"W	0	2
BA-210408	Riachão das Neves	11°50'48"S/45°74'763"W	0	0
BA-210409	Riachão das Neves	11°50'48"S/45°74'763"W	0	134
BA-210410	Riachão das Neves	11°30'48"S/45°43'186"W	22	0
BA-210411	Riachão das Neves	11°30'23"S/45°43'187"W	0	0
BA-211412	Jaborandi	14°17'51"S/45°27'048"W	14	0
BA-211413	Jaborandi	14°17'51"S/45°27'048"W	31	0
BA-211414	Jaborandi	14°17'56"S/45°26'999"W	0	19
BA-211415	Jaborandi	14°17'56"S/45°26'999"W	0	0
BA-211416	Jaborandi	14°17'77"S/45°27'512"W	29	0
BA-211417	Jaborandi	14°17'77"S/45°27'512"W	26	0
BA-210418	Riachão das Neves	11°39'40"S/45°43'520"W	15	0
BA-210419	Riachão das Neves	11°39'40"S/45°43'520"W	0	0
BA-210420	Riachão das Neves	11°40'13"S/45°42'786"W	51	37
BA-210421	Riachão das Neves	11°40'13"S/45°42'786"W	0	0
BA-210222	São Desidério	12°39'94"S/44°39'094"W	0	29
BA-211223	São Desidério	12°50'35"S/45°42'418"W	10	0
BA-210824[b]	São Desidério	12°49'24"S/45°15'150"W	643	0
BA-210825[b]	São Desidério	12°49'24"S/45°15'150"W	550	0
BA-210826[b]	São Desidério	12°51'49"S/46°16'419"W	2332	0
BA-211227	São Desidério	15°09'10"S/46°06'487"W	0	176
BA-210828	São Desidério	12°38'57"S/46°13'175"W	0	1209
BA-210829	São Desidério	12°37'91"S/46°11'147"W	0	0
BA-210830	São Desidério	12°51'38"S/46°08'923"W	0	15
BA-210831	São Desidério	12°51'30"S/46°09'520"W	0	0
BA-211132[b]	Correntina	13°36'40"S/46°02'587"W	8347	0
BA-211133[b]	Correntina	13°36'40"S/46°02'587"W	3859	0
BA-211234	São Desidério	13°01'11"S/45°52'474"W	0	0
BA-211235	São Desidério	13°00'10"S/45°54'119"W	15	0
BA-211236	São Desidério	13°00'15"S/45°54'157"W	32	3271

BA-211138	Correntina	13°13'58"S/45°22'107"W	12	2134
BA-211139	Correntina	13°14'26"S/45°22'468"W	16	0
BA-211140	São Desidério	13°09'34"S/46°07'155"W	0	1290
BA-211141	São Desidério	13°09'28"S/46°05'316"W	0	0
BA-211142[b]	Riachão das Neves	11°39'41"S/45°42'178"W	1127	0
BA-210843	São Desidério	12°36'38"S/46°16'431"W	12	0

[a] *M. enterolobii* and the others are *M. incognita.* [b] Populations selected for greenhouse experiment.

Later, in the same year, a total of 42 samples were evaluated for the presence of nematodes of the genus *Meloidogyne,* and the data are shown in Table 2. Juveniles of *Meloidogyne* sp. were found in 21 samples, occurring in 50% of the surveyed area. Six samples showed more than 500 specimens per 200 cm³ of soil plus 10 g of roots, three from the municipalities of São Desidério, Correntina and Riachão das Neves. Another important plant-parasitic nematode from cotton was also detected: second-stage juveniles of *Rotylenchulus reniformis* Linford and Oliveira, 1940, appearing in only 31% of the sampled area, mainly in the municipality of São Desidério.

3.2. Biochemical and molecular identification of Meloidogyne *species*

Using α-esterases (Est), the phenotype Est En2 was observed in the cotton samples collected in June 2021 in São Desidério (12°46'53" S/45°57'058"W), with two major bands (Rm 0.7, 0.9) and two secondary bands (Rm: 0.75, 0.95) (Fig. 5a). This esterase phenotype is typical of *M. enterolobii* (Carneiro *et al.*, 2016). The six other populations from western Bahia collected later (BA-210824, BA-210825, BA-210826, BA-211132, BA-211133 and BA- 211142) were all identified as *M. incognita,* showing the Est I2 phenotype with two bands, a major band (Rm:1.1) and a minor (Rm:1.2) (Fig. 5b), and *M. enterolobii* was not detected again in other samples. After reproduction on tomato plants and extractions of eggs and the genomic DNA, a conventional PCR was performed using MK7F/MK7R primer sets for *M. enterolobii* (Tigano *et al.*, 2010) and Inck14R/F for *M. incognita* (Randig *et al.*, 2002). Based on the specificity of the primers used and

the amplification size (520 bp), the species collected in June 2021 was also confirmed as *M. enterolobii* (Fig. 5c). The other six populations were identified as *M. incognita,* presenting an amplification of 399 bp (Fig. 5d).

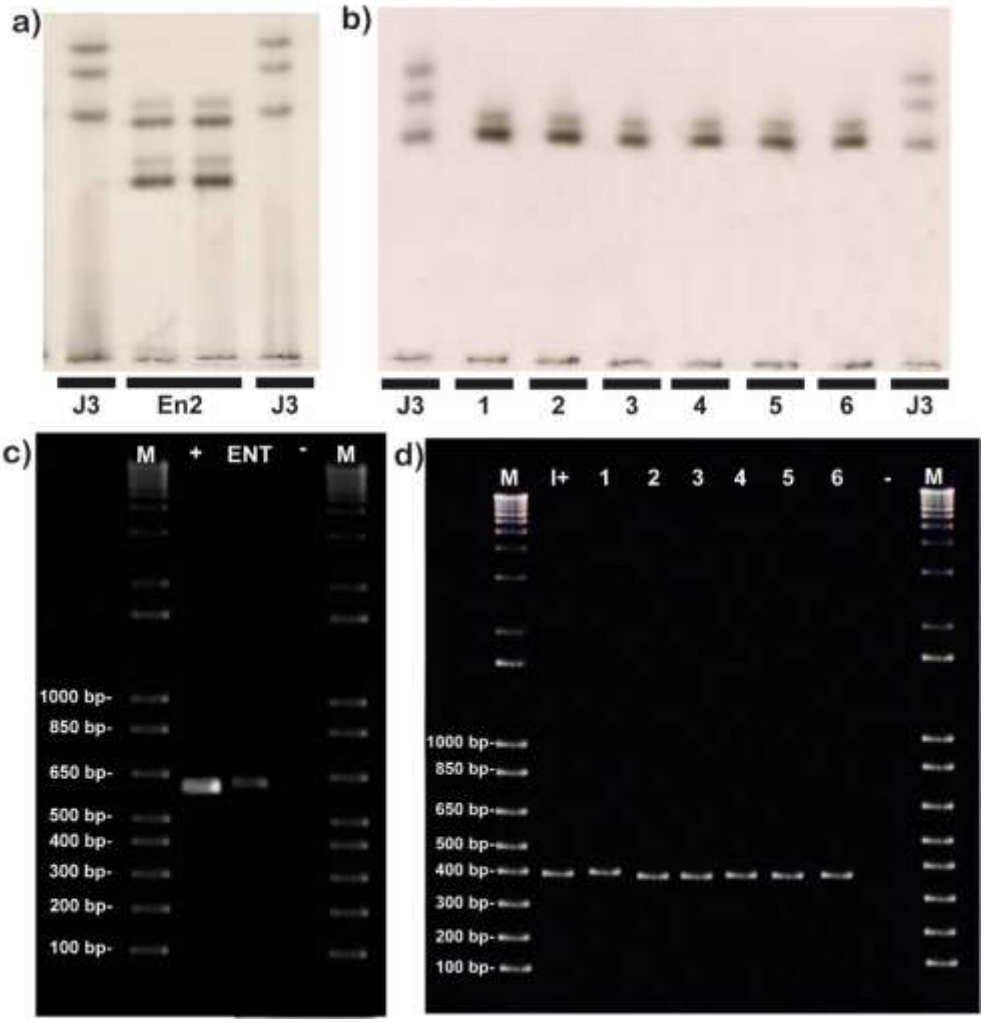

Figure 5 - a) Esterase phenotypes of *Meloidogyne enterolobii* (Est En_2) from cotton, Bahia state and *M. javanica* (Est J3) as pattern reference. b) Esterase phenotypes of *M. incognita* (EST I2) 1: BA-210824, 2: BA-210825, 3: BA-210826, 4: BA-211132, 5: BA-211133, 6: BA-211143. J3: *M. javanica* pattern (EST J3) included as reference. c) PCR amplification from eggs of two populations of *M. enterolobii*, using the SCAR primers MK7F/R (520 bp). (+) represents positive control (population from guava) and, ENT population from cotton (Bahia). M=1-kb Plus DNA Ladder. d) PCR amplification patterns of *M. incognita*: 399 bp generated with specific SCAR primers inc-K14-F/R where 1: BA-210824, 2: BA-210825, 3: BA-210826, 4: BA-211132, 5: BA-211133, 6: BA-211143. (I+): positive control for *M. incognita*, (-) DNA: negative control. M: 1-kb Plus DNA Ladder, bp: base pairs.

3.3. Reproduction of Meloidogyne enterolobii and M. incognita *populations on resistant and susceptible cotton cultivars*

For *M. enterolobii,* the greenhouse assay confirmed the pathogenicity of this nematode for cotton and the virulence in the resistant cultivar. In the cultivar TMG 44B2RF the RF (susceptible) ranged from 4.0 to 31.5, and in the cultivar IMA 5801B2RF from 11.0 to 15.8, evidencing the high reproduction of this population on cotton and its ability to overcome the resistance genes of cotton (*qMi-C11* and *qMi-C14*).

The greenhouse assay did not confirm the virulence of the populations of *M. incognita* collected in western Bahia. The reproduction factor (RF) in the resistant cultivar IMA 5801B2RF was less than 1 (0.10-0.47), confirming the highly resistant phenotype conferred by the two QTLs of resistance (*qMi-C11* and *qMi-C14*). In contrast, the susceptible control 'TMG 44B2RF' exhibited mean RF values of 19.9 (2.1-73.9) for the three tested populations. In conclusion, the populations of *M. incognita* evaluated in this bioassay were not virulent to the resistant cultivar IMA 5801B2RF.

4. Discussion

This is the first report of cotton plants infected by *M. enterolobii* in the state of Bahia, located more than 500 km from the initial point of detection of this nematode on cotton in Minas Gerais state (Galbieri *et al.*, 2020). In the USA, the population on cotton was found only in one region of North Carolina state. Populations of *M. enterolobii* from guava have been found in different Brazilian states, through the spread of infected guava seedlings, and are considered a serious problem for the guava crop in Brazil (Carneiro *et al*., 2021). According to Carneiro *et al*. (2006) and other nematologists (personal communications), the populations of *M. enterolobii* from guava did not parasitize cotton. Araújo Filho *et al*. (2016) detected some populations of *M. enterolobii* from tobacco in Santa Catarina state; all populations were similar to the guava race populations (hosts:

tobacco, tomato, watermelon and pepper). The cotton race was not detected in tobacco surveys either (Araújo Filho *et al.*, 2016).

Nematological analyses performed on the 42 samples allowed the detection of two species of nematodes commonly associated with cotton crops in Brazil: *M. incognita* and *R. reniformis.* The same was reported by Starr *et al*. (2005). The natural occurrence of *M. enterolobii* on cotton was never reported in previous surveys carried out by Perina *et al*. (2017), Lopes (2015), and Galbieri *et al*. (2014) in the states of Bahia and Mato Grosso, where only the species *M. incognita* was found parasitizing cotton roots.

The numbers of *M. incognita* second-stage juveniles (J2) found in the samples of São Desidério, Correntina and Riachão das Neves municipalities (>500 J2/cm³ of soil) were higher than expected for genotypes carrying resistance genes (Galbieri *et al.*, 2009; Wheeler *et al.*, 2014). This suggests that a population is possibly breaking the oligogenic resistance of cultivar IMA 5801B2RF. The high reproduction rates of *M. incognita* populations from western Bahia state in fields with the resistant cultivar, but not in the greenhouse experiment, indicated the absence of virulent populations, suggesting that the cotton cultivar planted was not IMA 5801B2RF, as reported by the farmers, probably due to incorrect field identification and/or seed mixture in on-farm seed production. More investigations are necessary to know what is happening in that region, but in any case, the occurrence of *M. enterolobii* in cotton seems to be restricted to only one area so far.

Although *M. incognita* populations breaking the resistance of genotypes with a single QTL such as 'Acala NemX' and 'LA 887' have already been described (Zhou *et al*., 2000), virulent populations with high rates of reproduction in genotypes with the two QTLs of resistance have not yet been found in cotton. According to Castagnone-Sereno (2002), Lindhout (2002) and Khallouk *et al*. (2013), when resistance has an oligo or polygenic origin, the genotype is less vulnerable to having its resistance overcome by *M.*

incognita populations. Our results showed the stability of resistance of the resistant cotton IMA 5801B2RF cultivar in the greenhouse, conferred by the expression of two genes of resistance, and this was mentioned before by Shepherd (1983) and Starr and Roberts (2004) as a way to prevent the appearance of virulent biotypes.

This first report of *M. enterolobii* is essential for local control measures and for preventing the spread of this nematode to other areas. The presence of this nematode in the state of Bahia may pose a threat to Brazilian cotton production, due to the ability of *M. enterolobii* to cause damage to this crop and considering that Bahia state contributes more than 20% of the Brazilian cotton production. More studies on races and intraspecific variability of *M. enterolobii* populations will be necessary to clarify the potential damage that this RKN species can cause in important commodities like cotton and soybean, among others.

5. Conclusions

The identification of *M. enterolobii* natural infection in a cotton field of Bahia State marks a significant concern due implications for the Brazilian cotton industry. This nematode species, known for its destructive potential and high virulence, poses a challenge to crop productivity and agricultural sustainability (*e.g.* guava decay in Brazil). Our study not only confirms the presence of *M. enterolobii* in cotton but also reveals its alarming ability to overcome resistance genes in cotton. This highlights the urgent need for proactive management strategies and vigilant monitoring to contain the spread of this nematode. *Meloidogune enterolobii* exhibited high reproduction rates, including resistant cotton cultivars, while *M. incognita* populations did not demonstrate virulence to the resistant cultivar tested. Evidencing an incorrect field identification and/or seed mixture in on-farm seed production. Further research on the races and variability of *M. enterolobii* populations will be crucial for understanding its impact on cotton and other important

crops. In conclusion, this study sheds light on the dynamics of nematode infestation in cotton. By elucidating the virulence patterns of *M. enterolobii* and *M. incognita*, this research provides valuable insights for sustainable pest management and crop breeding programs.

CHAPTER 3 - GENETIC DIVERSITY OF RACES OF *Meloidogyne enterolobii* CAUSING DAMAGE TO DIFFERENT CROPS IN BRAZIL

Abstract - This study investigated the genetic diversity and races of *Meloidogyne enterolobii* from Brazil, a significant threat to global agriculture with a broad host range. Genetic variability was assessed using 44 RAPD and 7 AFLP primers. The concatenated neighbor-joining analysis clustered two guava (race 1) and two cotton populations (race 2) with 95% and 100% bootstrap support, respectively. The two pepper populations clustered with the other populations of *M. enterolobii* race 1, and the sweet potato (race 1) population was the most divergent (36% of polymorphism) and clustered separated, but related to other race 1 populations. Despite RAPD and AFLP analysis, genetic traits linked to host races remain elusive. Mitochondrial (*COII*) study grouped the two populations race 2 (cotton from Brazil); ribosomal DNA (ITS, D2-D3), and HSP90 gene studies revealed low interactions related to hosts races and geographical origin. The North Carolina Differential Hosts Test (NCDHT) demonstrated distinct pathogenic profiles in five populations, supporting a subclassification of *M. enterolobii* into two physiological races: race 1 (positive reaction on tomato, tobacco, watermelon, and pepper but not on peanut and cotton) and race 2 (positive reaction on tomato, tobacco, watermelon, pepper, and cotton but not on peanut). Considering that the cultivars proposed by NCDHT are obsolete, we assessed the efficacy of current Brazilian cultivars which, exhibited potential as suitable alternatives for conducting *Meloidogyne* spp. race tests. None of the recently tested cultivars possess resistance genes, except for tobacco cv. NC4 which carries the Rk gene. The cultivars recommended are tomato 'Santa Clara', pepper 'Magali R', watermelon 'Crimson Sweet', peanut 'IAC Tatu', tobacco 'NC4' and cotton 'FM966'. This study emphasizes the importance of understanding intraspecific variability in

managing the impact of this nematode through genetic resistance and crop rotation strategies.

Keywords: *AFLP, COII,* cotton, differentiating cultivars guava nematode, host races, *RAPD*.

Resumo – DIVERSIDADE GENÉTICA DE RAÇAS DE *Meloidogyne enterolobii* CAUSANDO DANOS A DIFERENTES CULTURAS NO BRASIL

Este estudo investigou a diversidade genética de raças de populações brasileiras de *Meloidogyne enterolobii*, uma ameaça significativa para a agricultura global com uma ampla gama de hospedeiros. A variabilidade genética foi avaliada usando 44 primers RAPD e 7 AFLP. A análise de agrupamento concatenado de *neighbor-joining* agrupou duas populações de goiaba (raça 1) e duas populações de algodão (raça 2) com suporte de bootstrap de 95% e 100%, respectivamente. As duas populações de pimentão se agruparam com as outras populações de *M. enterolobii* raça 1, e a população de batata-doce (raça 1) foi a mais divergente (36% de polimorfismo) e agrupou separadamente das demais, mas se juntou com as outras populações da raça 1. Apesar dos agrupamentos da análise de RAPD e AFLP, os traços genéticos ligados às raças hospedeiras permanecem indefinidos. O estudo mitocondrial (*COII*) agrupou as duas populações brasileiras pertencentes a raça 2 (algodão); estudos de DNA ribossômico (*ITS*, *D2-D3*) e do gene *HSP90* revelaram pouca ou nenhuma interação relacionada às raças fisiológicas ou origem geográfica. O teste de hospedeiros diferenciadores da Carolina do Norte (NCDHT) demonstrou perfis patogênicos distintos em cinco populações, suportando uma subclassificação de *M. enterolobii* em duas raças fisiológicas: raça 1 (reação positiva em tomate, tabaco, melancia e pimentão, mas não em amendoim e algodão) e raça 2 (reação positiva em tomate, tabaco, melancia, pimentão e algodão, mas não em amendoim).

Considerando que as cultivares propostas pelo NCDHT estão obsoletas, avaliou-se a eficácia das cultivares atuais disponíveis no Brasil, que apresentaram potencial como alternativas para se conduzir testes de raças de *Meloidogyne* spp. Nenhuma das cultivares testadas possui genes de resistência, exceto o tabaco cv. NC4, que carrega o gene *Rk*. As cultivares recomendadas são tomate 'Santa Clara', pimentão 'Magali R', melancia 'Crimson Sweet', amendoim 'IAC Tatu', tabaco 'NC4' e algodão 'FM966'. Este estudo enfatiza a importância de entender a variabilidade intraespecífica no manejo do impacto deste nematoide por meio de resistência genética e estratégias de rotação de culturas.

Palavras-chave: *AFLP,* algodão, *COII,* cultivares diferenciadoras, nematoide da goiabeira, *RAPD*, raça fisiológica.

1. Introduction

Meloidogyne enterolobii Yang & Eisenback, 1983 (sin. *M. mayaguensis* Rammah & Hirschmann, 1988), known as guava root-knot nematode, is considered a risk to agriculture due to its worldwide distribution and high host range, reported in more than 67 species of cultivated plants, distributed in more than 27 botanical families (Castagnone-Sereno, 2012; Castillo & Castagnone-Sereno, 2020). This species is recognized as being among the most damaging root -knot nematode (RKN) species due to its ability to reproduce in host plants that carry resistance against major tropical RKNs (Castagnone-Sereno, 2012).

The species *M. enterolobii* was described in 1983 from a population causing severe damage to the pacara earpod tree (*Enterolobium contortisiliquum* (Vell.) Morong) in China (Yang and Eisenback, 1983). In 1988, a new species of the genus, named *M. mayaguensis* was described from a population from Puerto Rico, which was later synonymized as *M. enterolobii*, based on esterase (En2) patterns, mitochondrial DNA similarities and finally on morphological and morphometrical parameters (Xu *et al.*, 2004; Hunt and Handoo, 2009; Karssen *et al.*, 2012). It is worth mentioning that both species showed the same positive host response for tobacco, pepper, watermelon and tomato and no host response for peanut. Besides this, in the original description, *M. mayaguensis* did not infect cotton, while *M. enterolobii* infected this crop, suggesting the existence of different host reactions for these species, based on Hartman and Sasser (1985) methodology (Yang and Eisenback, 1983; Rammah and Hirschmann, 1988).

In recent years, *M. enterolobii* emerged as the primary nematological challenge affecting guava crops in several countries (Carneiro *et al*., 2021). Compared with other RKN species, *M. enterolobii* is not controlled by various sources of RKN resistance, which constitutes a challenge for controlling this species. For example, *M. enterolobii*

reproduced verry well on resistant cotton (*qMi-c11* and *qMi-c14* loci), sweet potato, tomatoes (*Mi*-gene), potato (*Mh* gene), soybean (*Mir1* gene), bell pepper (*N* gene), sweet pepper (*Tabasco* gene) and cowpea (*Rk* gene) (summarized in Castagnone-Sereno, 2012). Similarly, *M. enterolobii* has been found inducing severe root galling, plant defoliation, yield losses and reduction of fruit quality on *Capsicum* rootstock 'Snooker', carrying the *Me1* and *Me3*/*Me7* genes (Pinheiro *et al.*, 2015) in a commercial greenhouse in Brazil. Recently, *M. enterolobii* was detected on sweet potato in the state of Ceará, Brazil, causing serious damage to this crop (Silva *et al*., 2021), and confirming a previous detection made some years earlier on this crop and other vegetables (Silva *et al*., 2016).

Reports on *M. enterolobii* parasitizing cotton in Brazil have been documented in Minas Gerais and Bahia states. In both cases, the cotton plants carried two resistance genes to *M. incognita* (Kofoid & White, 1919) Chitwood, 1949, namely *qMi-C11* and *qMi-C14* (Galbieri *et al*., 2020; Souza *et al*., 2022). The affected plants exhibited reduced size and root galls, which were larger than those typically induced by *M. incognita* (Souza *et al.*, 2022). However, it is important to note that the distribution of *M. enterolobii* in Bahia state remains very limited until now, and no losses in cotton yields have been reported so far. On the other hand, the first detection of *M. enterolobii* on soybeans in Brazil occurred in Ituverava, São Paulo state, causing a significant threat to the infested farm (Almeida *et al.*, 2008). This species was also detected on soybean in the same area in Minas Gerais state, Brazil (Rafael Galbieri, personal communication).

In the USA, *M. enterolobii* was found naturally parasitizing ornamental plants (Brito *et al.*, 2004). In 2012, it was detected in the Goldsboro region, Wayne County, North Carolina, affecting cotton and soybean crops (Ye *et al.*, 2013). More recently, it was detected in resistant sweet potato crops in South Carolina, making a significant threat not only to this particular crop but also to other crops across the USA (Rutter *et al.*, 2019).

Meloidogyne species can be differentiated based on morphological descriptions, isozyme phenotypes (esterases, Est) and molecular analysis, which have been proven reliable tools, which allowed diagnostics in most cases (Esbenshade and Triantaphyllou, 1985; Brito *et al.*, 2004; Castagnone-Sereno *et al.*, 2011; Carneiro *et al.*, 2016; Carneiro, *et al.*, 2021). Within a given RKN species, races can be recognized according to their reproduction on different host plants, as standardized into the North Carolina differential host test (NCDHT) based on the study of nearly 1000 populations of the more common RKN species in the frame of the International *Meloidogyne* Project (Hartman and Sasser, 1985). It is based on six plant species and specific cultivars: tomato (*Solanum lycopersicum* L.'Rutgers'), sweet pepper (*Capsicum annuum* L. cv. 'California Wonder'), cotton (*Gossypuim hirsuntum* L.'DP 61'), peanut (*Arachis hypogaea* L. 'Florunner'), watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai 'Charleston Gray'), and tobacco (*Nicotiana tabacum* L. 'NC 95'). These cultivars were selected due to their absence of resistance genes to RKN, except for Tobacco 'NC 95', which contains the *Rk* gene providing resistance against races 1 and 3 of *M. incognita* (Hartman and Sasser, 1985; Subbotin *et al*., 2021). However, these cultivars were chosen for the test decades ago, and some of these seeds are no longer available in the market, making their acquisition very difficult.

Even though, the RKN host races exhibit physiological consistency, no genetic traits associated with these races have been identified. Okimoto *et al*. (1991) proposed a differentiation of host races of *M. javanica* (Treub, 1885) Chitwood, 1949 based on repeated sequence sets on the mitochondrial genome. However, this study only considered one population per race, which raises the possibility that the differentiation may be influenced by characteristics unrelated to the race. Subsequent studies employing RAPD and ISSR markers showed no relationship between these markers and host

response in many *Meloidogyne* species and populations (Cenis, 1993; Baum, 1994; Randig *et al.*, 2002; Santos *et al.*, 2012). More recently, the sequencing of the genome of four races of *M. incognita* (eleven isolates) did not reveal any correlation between genetic composition and host races, all isolates being genetically identical (Koutsovoulos *et al.*, 2020b). These differences in the host races are probably due to trans-generational epigenetic events and/or post-transcriptional gene regulation rather than genomic traits (Perfus-Barbeoch *et al.*, 2014; Castagnone-Sereno *et al.*, 2019; Koutsovoulos *et al.*, 2020a) However, the differential host test is consistent with the occurrence of *Meloidogyne* spp. races (Subbotin *et al*., 2021), and it is still largely used for the selection of resistant varieties in different crops (coffee, cotton, soybean and others). Populations of *M. enterolobii* (=*M. mayaguensis*) from guava were classified as non-parasites of cotton by Carneiro *et al*. (2006), and a new race was recently detected on resistant cotton in Brazil (Souza *et al.*, 2022). Studies of intraspecific variability of *Meloidogyne* spp. populations are essential when genetic resistance and crop rotation are considered the most appropriate control strategies. In this study, the genetic variability and relationships among *M. enterolobii* populations obtained from different crops and geographical regions were analysed based on molecular and physiological parameters to resolve the diversity of Brazilian *M. enterolobii* populations and their race status. To update the original NCDHT (Hartman and Sasser, 1985), new cultivars of different plants (tomato, sweet pepper, cotton, peanut, watermelon and tobacco) were tested and compared with the original ones and recommended as new host differential plants to be used in future studies.

2. Materials and methods

2.1.Nematode populations

All seven populations of *M. enterolobii* originated from Brazil (Table 3) from different crops and localities were maintained and multiplied under greenhouse conditions on tomato cv. Santa Clara plants. These populations were identified and purified by esterase (Est) phenotyping, according to Carneiro and Almeida (2001), and confirmed with SCAR-PCR marker (Tigano *et al.*, 2010). One population of *M. hapla* was included in the study as an outgroup for RAPD and AFLP analysis.

Table 3 - *Meloidogyne enterolobii* populations used in the study and Genbank accession number of your respective region sequences.

Code	Geographical origin[a]	Host/Crop	Genbank accession number			
			ITS	D2-D3	COII	Hsp90
Me7	Petrolina, PE, Brazil	Guava	OR515678	OR502878	OR538110	OR531089
Me9	São João da Barra, RJ, Brazil	Guava	OR515679	OR502879	OR538111	OR531090
Me13	Campos Novos Paulista, SP, Brazil	Pepper	OR515680	OR502880	OR538112	OR531091
Me14	Pirajú, SP, Brazil	Pepper	OR515681	OR502881	OR538113	OR531092
Me17	Jandaíra, RN, Brazil	Sweet potato	OR515682	OR502882	OR538114	OR531093
Me18	Paracatu, MG, Brazil	Cotton	OR515683	OR502883	OR538115	OR531094
Me19	São Desidério, BA, Brazil	Cotton	OR515684	OR502884	OR538116	OR531095

[a]Brazilian states: BA: Bahia; MG: Minas Gerais; PE: Pernambuco; RJ: Rio de Janeiro; SP: São Paulo; RN:Rio Grande do Norte.

2.2.DNA preparation

The extraction of 200–400 µl of eggs from each population was done according to Carneiro *et al*. (2004) and stored at –80°C until use. For genomic DNA extraction, eggs were frozen in liquid nitrogen and ground with a mortar and pestle. The DNA was purified

from the resulting powder by phenol–chloroform extraction (Randig *et al.*, 2002) and stored at -20°C.

2.3.RAPD analysis

RAPD-PCRs were performed in a 13 µl final volume containing 1.3 µl 10× PCR buffer (Cellco), 0.4 µl 10 µM primer, 2 µl 1.25 mM dNTPs (Cellco), 0.2 µl Taq DNA polymerase (5 U/ µl, Cellco) and 3 µl total genomic DNA (3 ng/µl) of each population. The 44 random 10-mer oligonucleotide primers (Operon Technologies). Amplifications were performed on a T100® Thermal Cycler (Bio Rad, Hercules, CA), using the following conditions: 5 min at 94°C; 40 cycles of 30 s at 94°C, 45 s at 36°C, 2 min at 70°C; and a final extension of 10 min at 70°C (Randig *et al.*, 2002). PCR products were separated by electrophoresis in a 1.5% (wt/vol) agarose gel, stained with ethidium bromide (0.3 µg/ml) and visualized under UV light. All RAPD analyses were repeated at least twice.

2.4. AFLP analysis

For each population, 1 µg of total genomic DNA was digested overnight at 37°C with EcoRI (15 U/µl; Invitrogen) and ligated to the specific adapters following the method of Suazo & Hall (1999). A series of seven random 22-mer primers (Integrated DNA Technologies) were used, consisting of the EcoRI adapter core sequence 5′-GACTGCGTACCAATTCAGT-3′ plus one of the three selective nucleotides (AGT, ACT, ATT, GGC, CAG, CCT or TCG). PCRs were performed in a 25 µl final volume containing 1 µl digested DNA (50 ng/ µl), 2.5 µl 10× PCR buffer without magnesium chloride (Cellco), 1 µl 50 mM MgCl2, 0.5 µl 10 mM dNTPs, 1 µl 10 µM primer and 0.3 µl Taq DNA polymerase (5 U/µl; Cellco). DNA was amplified using the T100® Thermal Cycler (Bio Rad, Hercules, CA), under the following cycling parameters: 1 min at 95°C; 37 cycles of 1 min at 94°C, 1 min at 56°C, 2 min 30 s at 72°C; and a final extension of 10 min at 72°C (Suazo and Hall, 1999). PCR products were separated by electrophoresis in

a 1.5% (wt/vol) agarose–synergel (0.7% agarose, 0.4% synergel; Diversified Biotech), stained with ethidium bromide (0.3 μg/ml) and photographed under UV light. The analysis was repeated at least twice.

2.5. Genetic diversity of Meloidogyne enterolobii *populations*

DNA fingerprints obtained with RAPD and AFLP markers were used to infer the genetic diversity of the seven populations of *M. enterolobii* plus one population of *M. hapla* used as an outgroup. For each marker, amplified bands were scored as present or absent from the digitized photographs of the gels and converted into a 0–1 binary matrix. Phylogenetic reconstruction was performed using the neighbor-joining (NJ) algorithm (Saitou and Nei, 1987) in PAUP* v. 4b10 (Swofford, 2002), considering the data as unordered with no weighting. Testing of node support for the resulting trees was performed on 1000 bootstrap replicates with a cut-off value of 50%. Because the two types of markers could be considered independent from one another, the two datasets were analysed in a global NJ analysis using the total evidence approach proposed by Huelsenbeck *et al.* (1996) with the same settings as for the individual NJ analyses.

2.6.PCR and sequencing

For the phylogenetic analyses, the DNA of the seven populations of *M. enterolobii* was amplified using the primers for the ITS1-5.8 S-ITS2 region of rRNA (primer set: forward 5′-TTGATTACGTCCCTGCCCTTT-3′ and reverse 5′-TCCTCCGCTAAATGATATG-3′; Schmitz *et al.*, 1998); the D2-D3 fragment of the 28S rRNA gene (primer set: forward 5′-ACAAAGTACCGTGAGGGAAAGTTG-3′ and reverse 5′-TCGGAAG GAACCAGCTACTA-3′; De Ley *et al.*, 1999); the mtDNA cytochrome c oxidase II (COII) gene (forward 5′-GGTCAATGTTCAGAAATTTGTGG-3′ and reverse 5′-TACCTTTGACCAATCACGCT-3′; Powers and Harris, 1993) using the PCR conditions described by Subbotin *et al.* (2000); and HSP90 (primer set: forward 5′-

GCYGATCTTGTYAACAACCYT GGAAC-3′ and reverse 5′-TCGAACATGTCAAAAGGAGC-3′) with PCR conditions according to Nischwitz *et al.* (2013). All amplicons were separated by electrophoresis on a 1.2% TBE agarose-gel (wt/vol). The amplified products were sequenced by Macrogen (Seoul, South Korea).

Sequences from *M. enterolobii* populations were aligned using ClustalW in MEGA v. 5.0.3 (Tamura *et al.*, 2011) and compared with other *M. enterolobii* retrieved sequences from the NCBI database. The program MrModeltest (Nylander, 2004) implemented in PAUP* was used to identify the bestfit models for each analysis. The phylogenetic trees were generated based on maximum-likelihood (ML) analysis in IQtree (Trifinopoulos *et al.*, 2016). The phylogram was bootstrapped 1000 times, and only support values above 50% were considered. All sequences obtained were deposited in the NCBI database, all the accession numbers are presented in Table 3.

2.7.Race determination of Meloidogyne enterolobii *populations*

Race determination was performed the North Carolina Differential Host Test (NCDHT) (Hartman and Sasser, 1985) with the following plants: cotton cv. Deltapine 61, tobacco cv. NC95, pepper cv. Early California Wonder, watermelon cv. Charleston Gray, peanut cv. Florunner and tomato cv. Rutgers to determine the host-race status. Additionally, was performed an assay with the same botanical species, however, alternative cultivars were selected: cotton cv. FiberMax 966, tobacco cv. NC4 (with *Rk* gene), pepper cv. Magali R, watermelon cv. Crimson Sweet, peanut cv. IAC Tatu and tomato cv. Santa Clara. These plants were inoculated with 5000 eggs and second-stage infective juveniles (J2) of *M. enterolobii* and maintained under greenhouse conditions at 25–28°C for 2 months, with watering and fertilization as needed. The evaluation was carried out in two stages. In the first, the roots were washed gently and were immersed in a Phloxin B solution at 15 mg/1 liter of water for 20 minutes for staining the external egg mass and quantification

of the gall index (GI) and egg-mass index (EMI) (index 1: 1–2 galls or egg masses; index 2: 3–10 galls or egg masses; index 3: 11–30 galls or egg masses; index 4: 31–100 galls or egg masses; and index 5: over 100 galls or egg masses per root system. Host- plant types that have an average gall and egg mass index of 2 or less are designated non host or resistant (-), host plant on which nematode reproduction is 3, 4 or 5 (grater then 2) were considered host (+) or susceptibles (Hartman and Sasser, 1985). The total eggs and J2 evaluated in the root system, were extracted according to the modified Hussey and Barker (1973) methodology, using 1% of NaOH. The number of eggs and eventual J2 in the suspension of each repetition were counted under an optical microscope with the aid of a Peters chamber. Subsequently, the Reproduction Factor (RF) was determined by dividing the Final Population (FP) by the Initial Population (IP = 5000) (Oostenbrink, 1966). The reaction of different plants to *M. enterolobii* populations were classified according to the reproduction criteria established by Taylor (1967) with modifications in order to distinguish the different levels. This criterion established host suitability/resistance and was defined as follows: very good host (VGH), 50-100 % of nematode reproduction (RF similar to the tomato control); good host (GH), nematode reproduction (RF) that is 25 to 50% of that on the susceptible control (tomato); intermediate host (IH), 10 to 25% of that on the susceptible control (tomato) ; bad host (BH), 1 to 10 % of that on the susceptible control; not host (NH), RF less than 1.0 and Immune (I), RF = 0. To calculate the suitability criteria, the RF of susceptible tomato was considered a susceptibility standard, considering it as 100% nematode reproduction.

3. Results

3.1. Characterization of nematode populations

All seven nematode populations were identified as *M. enterolobii* based on esterase phenotype (En 2) with two major bands (Rm 0.7, 0.9) and two minor bands (Rm: 0.75, 0.95) (Fig. 6a). Additionally, PCR amplifications were performed with MK7F/MK7R primers, and the specific *M. enterolobii* 520-bp amplified fragment was observed for all seven populations (Figure 6b).

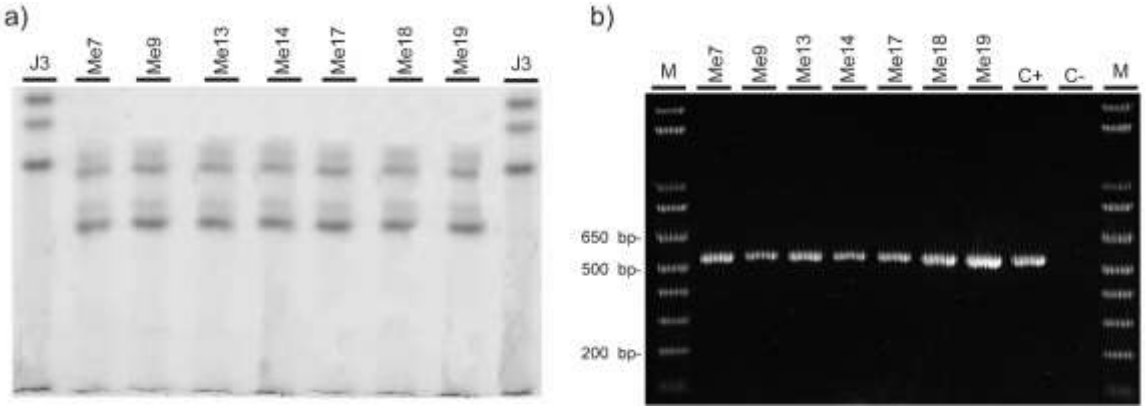

Figure 6 - a) Esterase phenotypes of *Meloidogyne enterolobii* (Est En4) populations, Me7 and Me9 from guava, Me13 and Me14 from pepper, Me 17 from sweet potato, Me18 and Me19 from cotton. *M. javanica* (Est J3) is included as a reference. b) PCR amplification from eggs of seven populations of *M. enterolobii*, using the SCAR primers MK7F/MK7R (520 bp) where Me7 and Me9 are from guava, Me13 and Me14 are from pepper, Me 17 is from sweet potato, Me18 and Me19 are from cotton. (+) represents positive control (population from guava), (−) DNA: negative control. M = 1-kb Plus DNA Ladder.

3.2. *Genetic diversity revealed by RAPD and AFLP markers*

The genetic variability of these seven *M. enterolobii* populations was assessed using RAPD and AFLP molecular markers. Overall, a total of 44 RAPD and 7 AFLP primers were used, and 440 reproducible amplified fragments were generated; their size ranged from ~150 to ~3800 bp, among which 55 (12.5%) were polymorphic, showing a rather low level of global polymorphism (Table 4). Pairwise comparisons revealed variable levels of polymorphism related to the original host of the nematode populations

and the two races. The two populations from guava and the two populations from cotton were very close together considering the same host with 7% and 6.75% polymorphism, respectively. Conversely, the two populations from pepper exhibited 19% polymorphism. The sweet potato population was the most divergent, with a polymorphism with the populations from other hosts ranging from 34 to 39.7%. Overall, the variability observed between populations from the same host was lower than between populations from different hosts (Table 4).

Table 4 - Characteristics of the molecular markers generated to evaluate the genetic diversity of *Meloidogyne enterolobii*.

	RAPD	AFLP
No. primers used	44	7
Amplification range (bp)	200-3800	150-3500
No. reproducible amplified fragments	189	251
No. polymorphic fragments (%)	32 (20.4)	23 (9.2)

The 0–1 binary matrix (absence/presence of fragments) obtained from the entire set of markers was used to infer the genetic relationships among the populations. The results from the NJ dendrogram (Fig. 7) confirmed a low intraspecific variability among *M. enterolobii* populations. The two cotton populations (Me18 and Me19) (race 2) on one side and the two guava populations (Me7 and Me 9) (race 1) on the other side clustered together, with 100% and 95% bootstrap support, respectively (Fig. 7). The populations of *M. enterolobii* race 1 from guava and pepper clustered also with high bootstrap support. The Me17 population from sweet potato (race1) also, stood phylogenetically far from the other populations with high percentage of polymorphic loci when compared with other populations (Table 5).

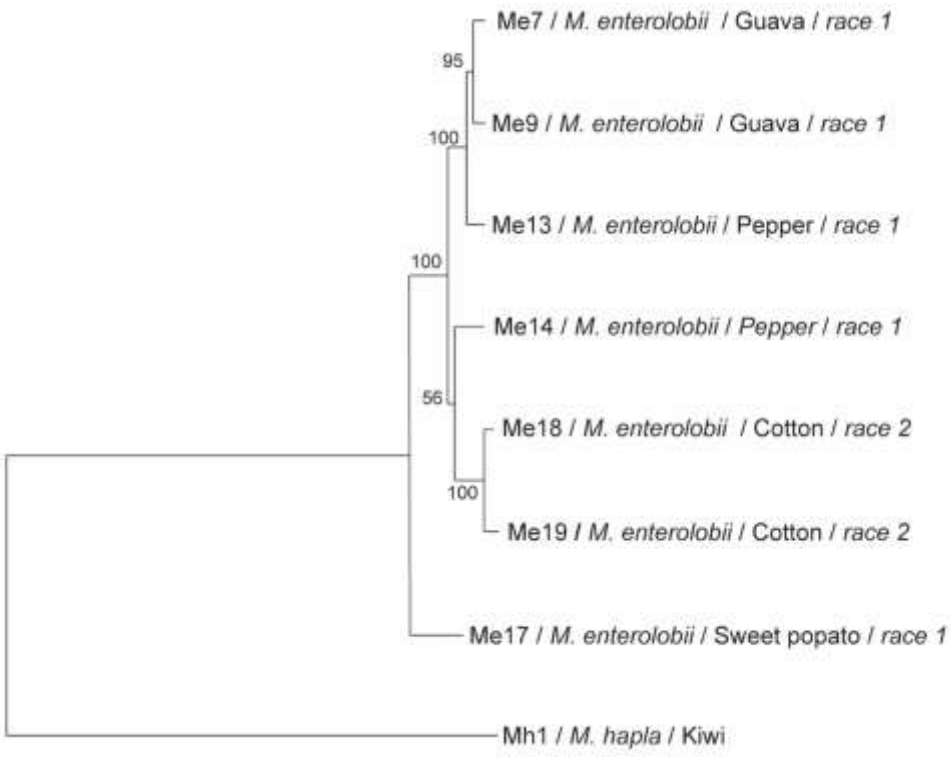

Figure 7 - Concatenated neighbour-joining tree showing the analysis of genetic variability (by random amplified polymorphic DNA [RAPD] and amplified fragment length polymorphism [AFLP]) of *Meloidogyne enterolobii* populations. Numbers to the left of the arms are the bootstrap values of 1000 replicates. Populations Me7 and Me9 are from guava, Me13 and Me14 are from pepper, Me 17 is from sweet potato, Me18 and Me19 are from cotton.

Table 5 - Number of AFLP and RAPD polymorphic loci between pairs of *Meloidogyne enterolobii* populations. The number in parenthesis represents the percentage of polymorphisms.

Population	Me7 Guava	Me9 Guava	Me13 Pepper	Me14 Pepper	Me17 Sweet potato	Me18 Cotton	Me19 Cotton
Me7 Guava	-						
Me9 Guava	27 (7.0)	-					
Me13 Pepper	39 (10.1)	38 (9.9)	-				
Me14 Pepper	72 (18.7)	71 (18.4)	73 (19.0)	-			
Me17 Sweet potato	145 (37.7)	148 (38.4)	134 (34.8)	131 (34.0)	-		
Me18 Cotton	92 (23.9)	89 (23.1)	91 (23.6)	80 (20.8)	143 (37.1)	-	
Me19 Cotton	98 (25.4)	90 (23.1)	99 (25.7)	76 (19.7)	153 (39.7)	26 (6.75)	-

3.3.Phylogenetic analysis

For each of the seven *M. enterolobii* populations studied, sequences homologous to the *ITS, D2-D3, COXII* and *HSP90* gene regions were amplified by PCR and fragments of approximately 680, 1000, 670 and 810bp were obtained, respectively (data not shown). The corresponding sequences were deposited in GenBank Sequence Database under the accession numbers listed in Table 1 and aligned with RKN homologous sequences available in databases. Whatever the gene considered, none of the phylogenetic reconstructions showed any clustering of sequences from all populations from same race or originating from the same host (Figs. 8 to 10). A slight exception was the tree constructed for the COXII, in which sequences from cotton populations (race 2) were grouped together according to the host and race. The same was observed for the *HSP90* gene for the guava populations (race 1). But in both cases, it should be noted that these populations were collected in Brazil.

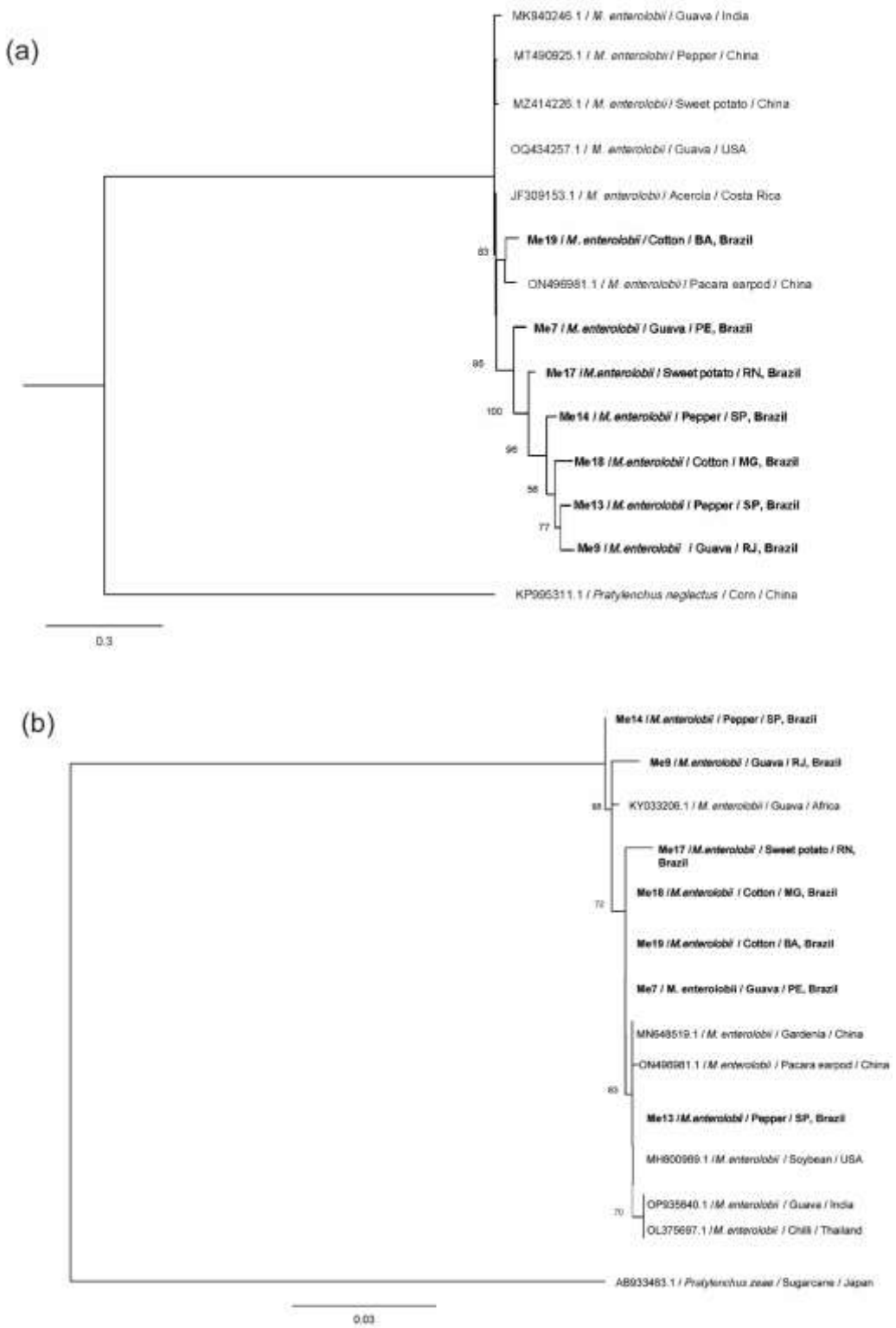

Figure 8 - Maximum-likelihood analysis showing the phylogenetic relationships of *Meloidogyne enterolobii* populations with related species based on the ITS1-5.8S-ITS2 rRNA sequences (a) and on the D2-D3 fragment of 28S rRNA sequences (b). Numbers to the left of the branches are bootstrap values for 1000 replications. Accession numbers for gene sequences retrieved from GenBank, and their respective host and country were listed adjacent to species names. Sequences from populations investigated in this study are highlighted in bold, with the addition of a population code, Me7 and Me9 are from

guava, Me13 and Me14 are from pepper, Me 17 is from sweet potato, Me18 and Me19 are from cotton.

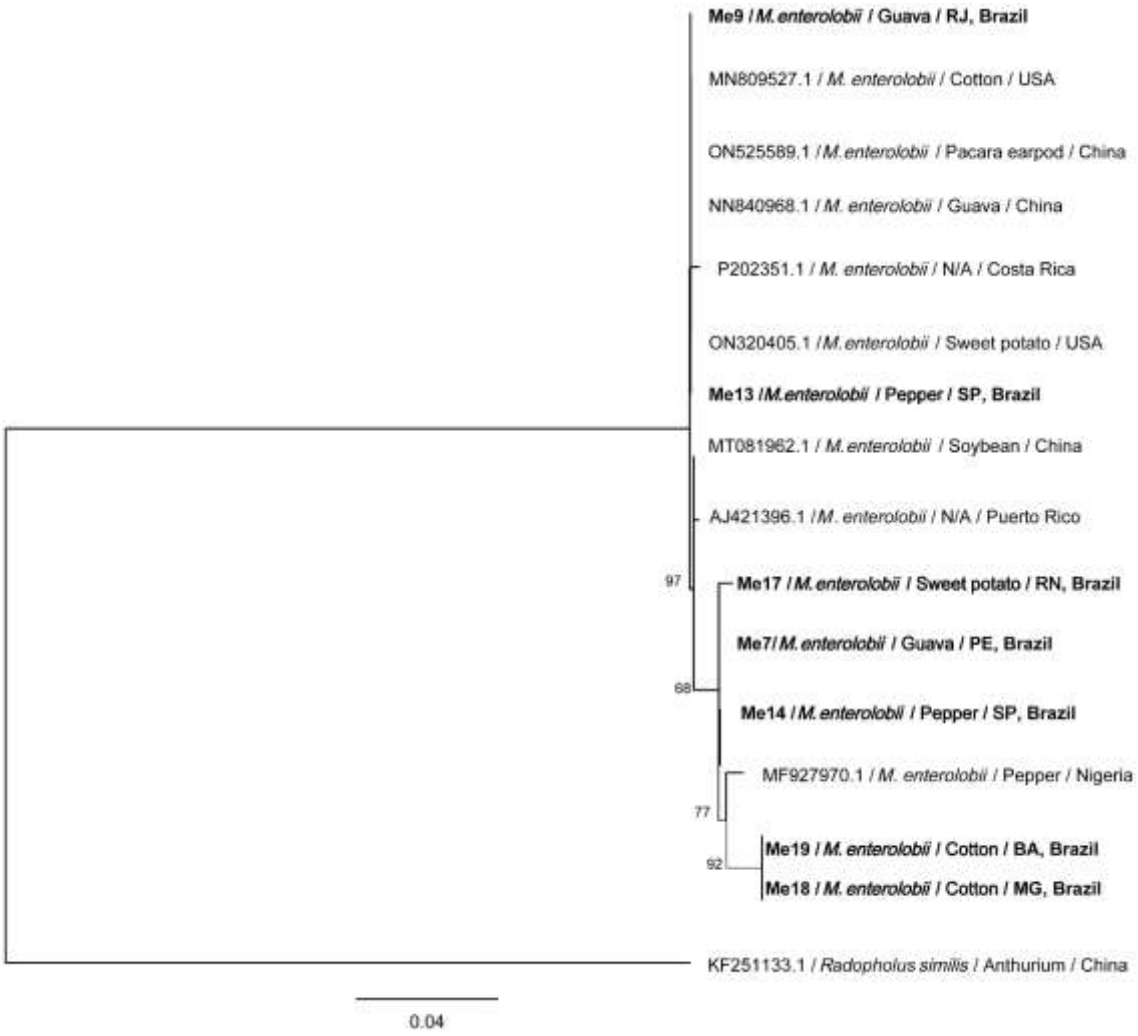

Figure 9 - Maximum-likelihood analysis showing the phylogenetic relationships of *Meloidogyne enterolobii* populations with related species based on the *COII*. Numbers to the left of the branches are bootstrap values for 1000 replications. Accession numbers for gene sequences retrieved from GenBank, and their respective host and country were listed adjacent to species names. Sequences from populations investigated in this study are highlighted in bold, with the addition of a population code, Me7 and Me9 are from guava, Me13 and Me14 are from pepper, Me 17 is from sweet potato, Me18 and Me19 are from cotton.

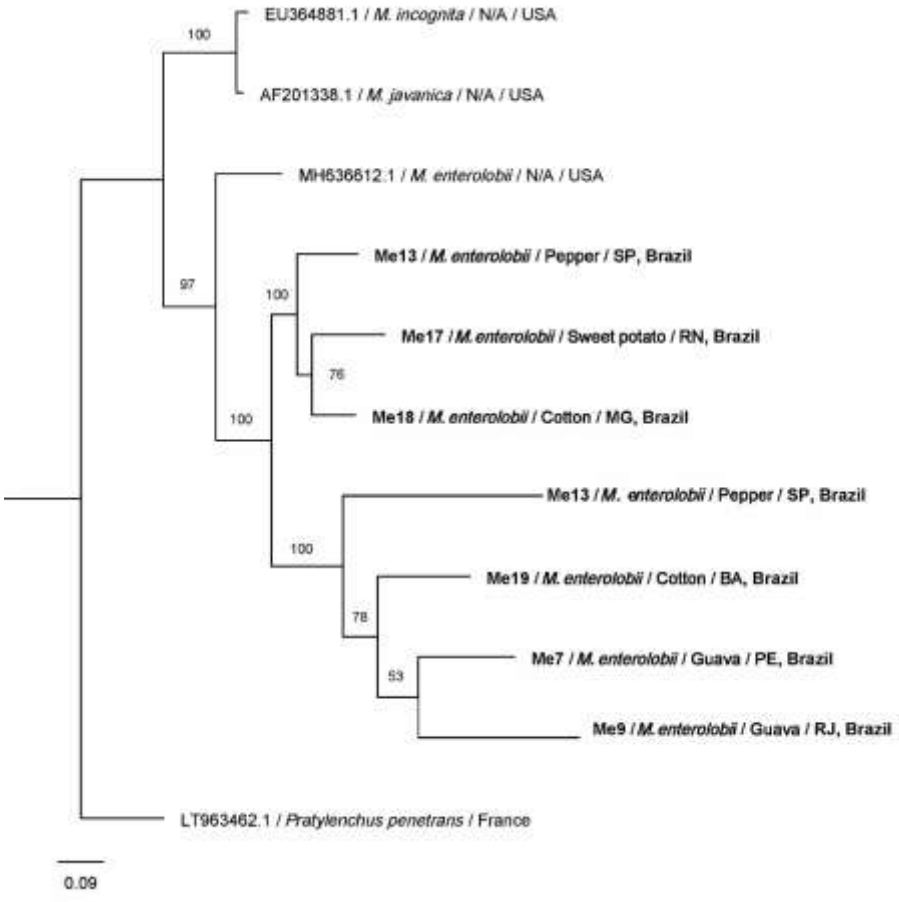

Figure 10 - Maximum-likelihood analysis showing the phylogenetic relationships of *Meloidogyne enterolobii* populations with related species based on the *Hsp90* gene sequences. Numbers to the left of the branches are bootstrap values for 1000 replications. Accession numbers for gene sequences retrieved from GenBank, and their respective host and country were listed adjacent to species names. Sequences from populations investigated in this study are highlighted in bold, with the addition of a population code, Me7 and Me9 are from guava, Me13 and Me14 are from pepper, Me 17 is from sweet potato, Me18 and Me19 are from cotton. N/A= Host not available.

3.4.Race determination of Meloidogyne enterolobii *populations using North Carolina Differential Host Test (NCDHT) and Taylor's criterion for original and current cultivars.*

The findings outlined in Tables 6 and 7 demonstrate a perfect correlation between the criteria of Taylor, 1967 and those of Hartman and Sasser (1985). All plants classified by both criteria as non-host (NH) and immune (I) were considered non host (-) and plants

classified as very good host (VGH), good host (GH) and intermediate host (IH) were good hosts (+) (Tables 6 and 7). The two tests are equivalent for determining differential hosts, being Taylor´s criterion more accurate as it quantifies the nematode reproduction (RFs), instead of establishing only indices of galls (GI) and egg masses (EMI). Three populations demonstrated a positive reaction on tomato, watermelon, pepper and tobacco, and a negative reaction on peanut and cotton (Table 6) using the NCDHT and the reproduction factors according Taylor's criteria (Table 6 and 7). The populations from guava (Me7), pepper (Me14) and sweet potato (Me17) were considered as race 1 since they did not parasitize cotton cultivars. Two populations (Me18 and Me19) reproduced on cotton, showing high gall and egg mass indices, indicating that these populations were considered as race 2 of *M. enterolobii.* The populations Me9 (guava) and Me13 (pepper) and other populations not included in this study were previously studied by Carneiro (personal information) and were classified as race 1 using only the NCDHT and were not included again in this study.

Table 6 - Gall index (GI) and egg-mass index (EMI) of differential hosts plants inoculated with 5000 eggs of five populations of *Meloidogyne enterolobii*. The classification was made following Hartman and Sasser (1985) criteria.

		Me7[a]		Me14		Me17		Me18		Me19	
Plant	Cultivar	GI	EMI	GI	EMI	GI	EMI	GI	EMI	GI	EMI
Cotton	DP 61	1.2 (-)[c]	0.4 (-)	1.4 (-)	0.8 (-)	1.6 (-)	0.4 (-)	5.0 (+)	5.0 (+)	5.0 (+)	5.0 (+)
	FM 966	0.6 (-)	0.2 (-)	0.4 (-)	0.0 (-)	1.2 (-)	0.4 (-)	5.0 (+)	5.0 (+)	5.0 (+)	5.0 (+)
Tobacco	NC 95	4.8 (+)	4.8 (+)	5.0 (+)	5.0 (+)	5.0 (+)	4.8 (+)	4.8 (+)	5.0 (+)	5.0 (+)	4.0 (+)
	NC 4	4.0 (+)	4.0 (+)	5.0 (+)	5.0 (+)	4.2 (+)	4.2 (+)	4.2 (+)	4.2 (+)	4.0 (+)	5.0 (+)
Pepper	California Wonder	5.0 (+)	5.0 (+)	5.0 (+)	5.0 (+)	5.0 (+)	5.0 (+)	5.0 (+)	5.0 (+)	5.0 (+)	5.0 (+)
	Magali R	5.0 (+)	5.0 (+)	5.0 (+)	5.0 (+)	5.0 (+)	5.0 (+)	5.0 (+)	5.0 (+)	5.0 (+)	5.0 (-)
Peanut	Florunner	0.0 (-)	0.0 (-)	0.0 (-)	0.0 (-)	0.0 (-)	0.0 (-)	0.0 (-)	0.0 (-)	0.0 (-)	0.0 (-)
	IAC Tatu	0.0 (-)	0.0 (-)	0.0 (-)	0.0 (-)	0.0 (-)	0.0 (-)	0.0 (-)	0.0 (-)	0.0 (-)	0.0 (+)
Watermelon	Charleston Gray	4.8 (+)	5.0 (+)	5.0 (+)	5.0 (+)	4.8 (+)	4.8 (+)	5.0 (+)	5.0 (+)	5.0 (+)	4.6 (+)
	Crimson Sweet	5.0 (+)	4.8 (+)	4.8 (+)	5.0 (+)	4.8 (+)	4.8 (+)	4.8 (+)	5.0 (+)	4.8 (+)	5.0 (+)
Tomato	Rutgers	5.0 (+)	5.0 (+)	5.0 (+)	5.0 (+)	5.0 (+)	5.0 (+)	5.0 (+)	5.0 (+)	5.0 (+)	5.0 (+)
	Santa Clara	5.0 (+)	5.0 (+)	5.0 (+)	5.0 (+)	5.0 (+)	5.0 (+)	5.0 (+)	5.0 (+)	5.0 (+)	5.0 (+)

[a]Populations details were provided in Table 1. [b]Gall and egg-mass indices following Hartman & Sasser (1985) classification; + = host, - = non-host.

Table 7 - Reaction of differential host plants inoculated with 5000 eggs of five populations of *Meloidogyne enterolobii*, based on the mean reproduction factor (RF) of different host cultivars and classified according to the criteria established by Taylor (1967) and Hartman & Sasser (1985).

Differential hosts	Population code[a]	Fresh root weight (g)	Eggs per g of roots	Reproduction Factor	Reaction[d]
Cotton ('DP 61' + 'FM 966')	Me7	25.1 a[b]	4.3 a	0.0 a	NH (-)
	Me14	32.2 a	3.3 a	0.0 a	NH (-)
	Me17	21.5 a	30.7 a	0.1 a	NH (-)
	Me18	33.5 a	2958.4 b	18.4 b	VGH (+)
	Me19	27.2 a	3404.5 b	32.7 c	VGH (+)
Pepper ('California Wonder' + 'Magali R')	Me7	97.6 a	1933.8 a	36.7 a	VGH (+)
	Me14	132.1 b	1567.6 a	41.3 a	VGH (+)
	Me17	130.2 b	1884.6 a	52.8 a	VGH (+)
	Me18	122.9 b	3322.7 b	84.0 b	VGH (+)
	Me19	109.3 a	3592.2 b	116.2 c	VGH (+)
Peanut ('Florunner' + 'Tatu')	Me7	29.3 b	0.0 a	0.0 a	I (-)
	Me14	34.7 b	0.0 a	0.0 a	I (-)
	Me17	9.7 a	0.0 a	0.0 a	I (-)
	Me18	18.1 a	4.7 b	0.0 a	NH (-)
	Me19	12.3 a	3.1 b	0.0 a	NH (-)
Watermelon ('Charleston Gray' + 'Crimson Sweet')	Me7	14.2 a	2286.9 a	6.2 a	VGH (+)
	Me14	19.3 a	1572.1 a	5.1 a	VGH (+)
	Me17	14.4 a	2946.8 b	3.8 a	IH (+)
	Me18	18.4 a	1866.6 a	6.7 a	GH (+)
	Me19	11.7 a	3229.3 b	10.1 a	VGH (+)
Tobacco ('NC 95' + 'NC 4')	Me7	214.8 b	156.1 a	5.5 a	GH (+)
	Me14	211.7 b	538.1 a	20.5 b	VGH (+)
	Me17	177.1 a	335.3 a	9.9 a	VGH (+)
	Me18	208.8 b	326.5 a	11.1 a	VGH (+)
	Me19	149.8 a	322.8 a	8.8 a	VGH (+)
Tomato ('Rutgers' + 'Santa Clara')	Me7	63.1 a	1107.7 a	13.8 a	VGH (+)
	Me14	65.6 a	719.2 a	9.3 a	VGH (+)
	Me17	67.6 a	1628.2 a	22.1 b	VGH (+)
	Me18	77.4 a	1083.0 a	16.1 b	VGH (+)
	Me19	60.7 a	1338.8 a	21.6 b	VGH (+)
CV (%)[c]		18.9	36.0	27.7	-

[a]Population details were provided in Table 1. [b]Means followed by the same lowercase letter in the column do not differ statistically from each other in the same host plant according to the Scott-Knott test at 5% probability. The mean values were transformed to $\sqrt{x+1}$ and the original data are presented in the table. [c]CV = Coefficient of variation after transformation. [d]Classification criteria proposed by Taylor (1967) with modifications, where VGH: very good host, GH: good host, IH: intermediate host; BH: bad host, NH: non host and I: immune; and Hartman & Sasser (1985) classification; + = host, - = non-host.

Additionally, we conducted a comparison between the original crop cultivars proposed by the NCDHT (Hartman and Sasser, 1985) with the cultivars currently available in the seed market in Brazil. In most plant species, there are no significant differences between the original cultivars and the new cultivars proposed in this study (Fig. 11a-f). However, there are a few variations. In the case of tobacco, the original NCDHT cultivar NC 95 displayed a higher nematode reproduction factor for the pepper and the sweet potato populations (Me 14 and Me17, respectively) than the new cultivar NC4 (Fig. 11e), but these values did not compromise the reactions of the differential host test when we compare the original and new cultivars, highlighting that the use of new cultivars does not invalidate the test proposed by the International *Meloidogyne* Project (Tables 6 and 7). Additionally, in the case of cotton, cultivar FM 966 exhibited significantly greater reproduction compared to 'DP 61' for the cotton race populations (Fig. 11f), showing that 'FM966' is a suitable substitute. Tomato plants were adopted as a standard of susceptibility in both criteria, and all populations were able to reproduce in this plant with reproduction factors (RF) greater than nine. However, variations in the RF were observed, and the guava (Me 7) and pepper (Me 14) populations exhibited significantly lower RFs than other populations (Table 7). All populations demonstrated markedly high RFs (36.7 – 116.2) on pepper plants, clearly establishing pepper as a preferential host for *M. enterolobii* among all tested host plants (Table 7). The cotton population from Bahia state (Me19) displayed a statistically significant rise in nematode reproduction (RF = 116.2), making the highest recorded reproduction factor. Following

closely, the cotton population from Minas Gerais state (Me18) exhibited the second-highest reproduction factor, RF = 84.0. Only the two cotton populations (Me18 and Me19) exhibited the ability to reproduce on cotton plants ('DP61' and 'FM966'), with RFs ranging from 18.4 to 32.7, categorizing them as very good hosts (VGH) for these populations. Conversely, other populations were deemed non-hosts (NH) (Table 7). On peanut, reproduction factors were nearly zero across all populations. However, for the two cotton populations, very low reproduction (3.1 – 4.7 eggs per gram of root) was observed. In these cases, peanut plants were classified as non-host (NH) and not as immune (I), unlike for other populations.

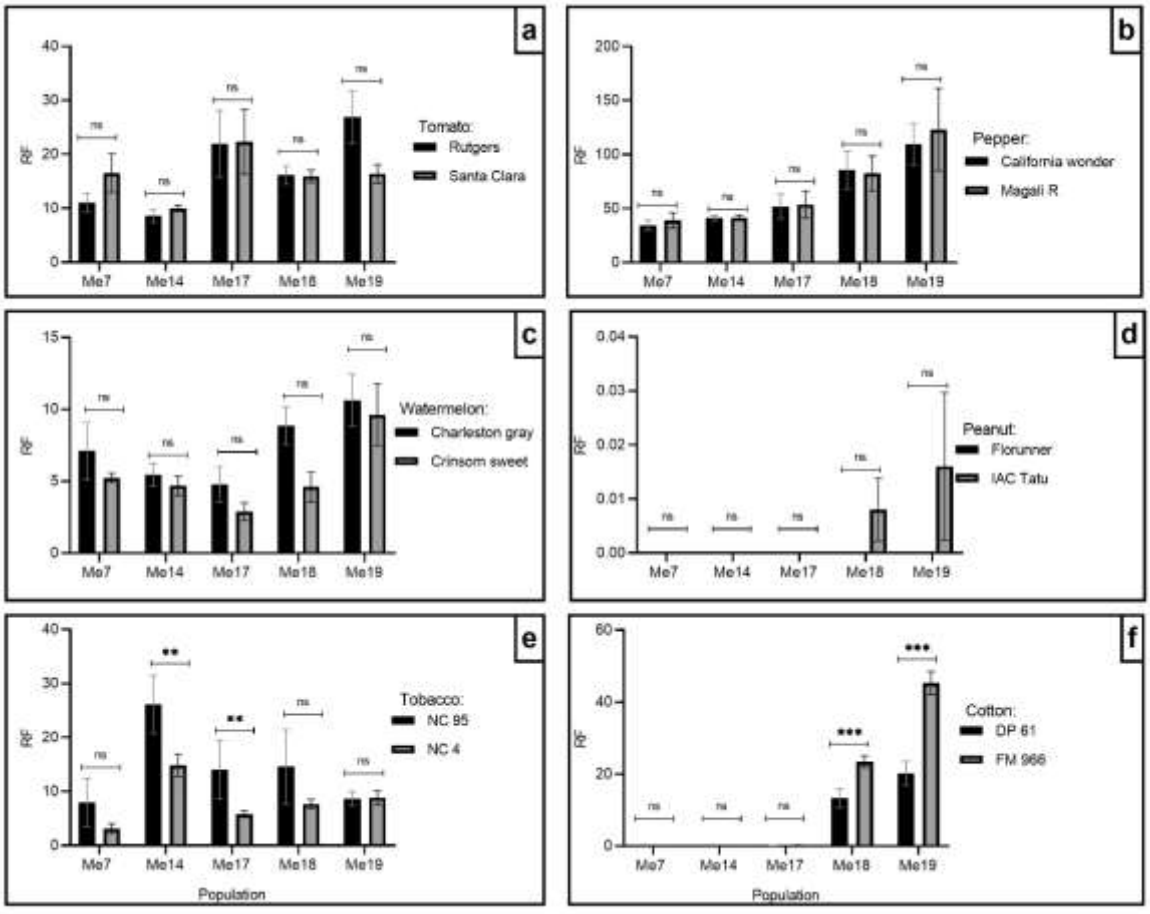

Figure 11 - Side-by-side comparison of reproduction factor (RF) of cultivar sets employed in the host circle test, inoculated with 5000 eggs of *Meloidogyne enterolobii*

populations (Me7 and Me9 are from guava, Me13 and Me14 are from pepper, Me 17 is from sweet potato, Me18 and Me19 are from cotton), where: a) tomato cultivars, b) pepper cultivars, c) watermelon cultivars, d) peanut cultivars, e) tobacco cultivars, and f) cotton cultivars. Asterisks denote the significance levels for cultivar comparisons: ** indicates P-value < 0.01, *** indicates P-value < 0.001, and "ns" denotes non-significance.

4. Discussion

The specific identification of populations used in this study was made based on their typical isozyme phenotype and PCR amplifications. All populations of *M. enterolobii* exhibited the same phenotypes for esterase (Est En2) (Esbenshade and Triantaphyllou, 1985; Carneiro *et al.*, 2016) for all host races. These phenotypes were reported first for *M. enterolobii* from China (Esbenshade and Triantaphyllou, 1985) and later for several populations of this species identified previously as *M. mayaguensis* (Fargette, 1987; Fargette and Waugh, 1996; Carneiro *et al*., 2000; Carneiro *et al.*, 2001; Brito *et al.*, 2004; Molinari *et al.*, 2005; Siqueira *et al.*, 2009). For DNA amplification by a specific SCAR marker all populations reached the amplification of 520 bp fragment, showing the SCAR marker MK7F/MK7R (Tigano *et al.*, 2010) is species-specific for *M. enterolobii* even in an intraspecific level as races.

This study reports first time the genetic diversity of *M. enterolobii* host races. The neutral molecular markers used here (AFLP and RAPD), allowed the evaluation of polymorphism among these populations. Our results shown a low diversity within species, confirming that *M. enterolobii* is a genetically homogenous RKN species, this observation could be linked in part to the obligatory mitotic parthenogenetic mode of reproduction of this nematode, where the egg develops directly into an embryo without any fertilization event (Yang and Eisenback, 1983). The low diversity in the RAPD marker of *M. enterolobii* in this work (20.4%) agrees with the results of Tigano *et al*.

(2010) where 16 *M. enterolobii* isolates from different geographical regions (Brazil and other countries) and hosts were used. This low intraspecific variability of *Meloidogyne* spp. was previously remarked also by Fargette and Waugh (1996) studying RLFPs of DNA of six West African lines of *M. enterolobii* (= *M. mayaguensis*) with similarities of more than 98.5% within this group of tropical resistance-breaking nematode.

The cotton race of *M. enterolobii* seems to have a very limited distribution in Brazil and in the USA (Ye *et al.*, 2013; Souza *et al.*, 2022); Araújo-Filho *et al.*(2016) studied 10 populations of *M. enterolobii* from tobacco in Brazil, none of them reproduced on cotton. Unfortunately, it was not possible to obtain other cotton populations from other country origins for our study, due to obstacles in quarantine regulations in Brazil and the USA. These two populations from cotton joined with 100% bootstrap support, clustering in the concatenated neighbor-joining tree (RAPD and AFLP) (Me18 and Me19) and 95% for guava populations (Me7 and Me9). However, considering the limited number of populations included, it would be premature to make assertions that clustering populations from cotton and other crops are related to the host status or race itself.

The analysis of *COII mtDNA* provides a rich source of genetic markers for identification in *Meloidogyne* spp. (Hu and Gasser, 2006; Blok and Powers, 2009), showing potential as a barcode region for distinguishing between RKN groups (Okimoto *et al.*, 1991; Hugall *et al.*, 1994; Pagan *et al.*, 2015). The *COII* gene region confirmed the results achieved with neutral molecular markers for the two Brazilian cotton *M. enterolobii* populations (race 2) grouped with 92% bootstrap support, but these findings cannot be extrapolated to all populations. The cotton population from the USA (MN809527.1) is an example. In addition, the intraspecific variation and genetic structure for *ITS*, *D2-D3* and *HSP90* were not well characterised for *M. enterolobii* populations and races.

The results of the host tests showed substantial consistency between the populations from guava, pepper, and sweet potato (race 1) and the populations from cotton (race 2). These findings align with previously reported results in the original description of *M. enterolobii* host (equivalent to race 4 of *M. incognita*) and *M. mayaguensis* host (equivalent to race 2 of *M. incognita*) (Yang and Eisenback, 1983; Rammah and Hirschmann, 1988). Rutter *et al.* (2021) identified a pathotypic variation in *M. enterolobii* between two isolates from the United States, where the isolate from North Carolina parasitized cotton, but the isolate from South Carolina did not. In this case, the authors classified these isolates as distinct pathotypes; however, the study included only one isolate per pathotype.

Moens *et al.* (2009) suggested that the formal recognition of the host races should be discontinued since the host race concept has sometimes been controversial, although the recognition of variation in host ranges is important for resistance tests (Lopes *et al.*, 2019). Gopal *et al*. (2022) tested 10 different host plants in an attempt to separate new races of *M. enterolobbii*. Despite the notably low reproduction factors, certain crops such as corn, sunflower, carrots, cotton, fodder turnips and phacelia proved intriguing, as they distinguished between multiple populations of *M. enterolobii* based on host response. Other crops like cucumber, eggplant, pepper, soybean, tobacco, tomato, bean, melon, potato, sugar beet and yellow mustard were considered good hosts. But the classification in different races was not clear because the RFs were low. Genetic sequencing studies of these different populations of *M. enterolobii* are currently underway (Etienne Danchin, personal communication). Nevertheless, given the variability within the genus *Meloidogyne*, it may not be possible to develop a single scheme to characterise the reactions of all nematode species to a wide range of crops throughout the world (Stanton and O'Donnell, 1998; Moens *et al*., 2009). Nonetheless, in our view, it can be

accomplished for various populations of a single species such as *M. enterolobii* that have been previously well identified using integrative taxonomy (Subbotin *et al*., 2021).

In our study, the traditional concept of host status clearly characterized the two races of *M. enterolobii* from Brazil (races 1 and 2), either using Hartman and Sasser (1985) or Taylor's (1967) criteria. The knowledge of races can also be applied in control strategies. The revelation that the *M.enterolobii* guava populations, the most spread in Brazil (Carneiro *et al*., 2021) do not parasitize cotton and peanut plants is highly significant, as it enables the use of these important crops in *M. enterolobii* infested areas. Knowledge on host races can also be used in plant breeding programs and other management strategies in the field (Subbotin *et al*., 2021).

A study with different Brazilian soybean varieties showed that the *M. enterolobii* population from cotton (race 2) was more aggressive than the guava population (race 1) (Verssiani *et al.*, 2023). The differences in aggressiveness observed among *M. enterolobii* populations are likely due to genetic variability within these populations. Variations in the aggressiveness of populations collected from diverse locations and hosts have been discussed in previous studies involving different *Meloidogyne* species sharing the same mode of reproduction as *M. enterolobii* (mitotic parthenogenesis) (Carpenter and Lewis, 1991; Castagnone-Sereno *et al.*, 1993; Medina *et al.*, 2017; Santos *et al.*, 2018a; Lopes *et al.*, 2019). Even so, no correlation could be found between cytogenetic, isoenzymatic, or molecular infraspecific polymorphism and the host in *Meloidogyne* species (Triantaphyllou, 1985; Cenis, 1993; Baum, 1994; Randig *et al.*, 2002; Santos *et al.*, 2012; Koutsovoulos *et al.*, 2020b). This observation raises questions on the genetic basis of such races, which probably do not represent monophyletic groups but rather a result of convergent evolution (Castagnone-Sereno *et al.*, 2013).

We emphasize that the replacement cultivars chosen alternatives to those recommended in Hartman and Sasser (1985) must belong to the same botanical species and should not harbour RKN resistance genes, except in the case of tobacco, the *Rk* gene is necessary, in order to avoid inconsistent reactions within the host range. All cultivars selected in this study as alternatives for the NCDHT proved to be appropriate for host race characterization, as evidenced by similar gall indexes and reproduction factors. It is worth noting that in the case of tobacco, the original cultivar NC95 allowed greater nematode reproduction, while in cotton, the alternative cultivar FM 966 exhibited higher nematode reproduction. Nonetheless, these variations did not impact the determination of nematode races, according to results presented in our study.

Considering the requirements of RKN race determination for studies of genetic resistance in plant breeding programs on crops of economic importance in Brazil (coffee, soybean, cotton, etc.) and, the updated knowledge of the genetic background of the different plants and cultivars used in NCDHT (Hartman and Sasser, 1985), it is no longer necessary to produce the seeds of obsolete cultivars recommended by the International *Meloidogyne* Project. Seeds of RKN currently susceptible cultivars of tomato, watermelon, pepper, cotton and peanut marketed in Brazil and in other countries can be used, with special attention to the tobacco cultivars with the resistance *Rk* gene, aimed at distinguishing between races of *M. incognita*.

5. Conclusions

In conclusion, this study elucidates the genetic diversity and race determination of *M. enterolobii* populations. Through AFLP, RAPD and sequences analysis (ITS2, D2-D3, COII and HSP90), this research provides conclusive evidence of low intraspecific variability among *M. enterolobii* populations, suggesting a genetically homogeneous species. The delineation of two distinct races based on differential host tests is a critical

step in understanding their behavior and devising effective management strategies. The findings not only validate traditional criteria for race determination but also highlight the need for updated host cultivars in such tests. By comparing original and current cultivars, this study demonstrates the suitability of modern cultivars for accurate race identification. As the genetic basis of host races remains enigmatic, further research into the underlying mechanisms driving these variations is necessary.

CHAPTER 4 - PARTIAL RESISTANCE ON COTTON TO *Meloidogyne enterolobii*: RESIDUAL EFFECT OF KNOWN QTLS AND A NEW SOURCE OF RESISTANCE IN REDUCING NEMATODE REPRODUCTION.

Abstract - *Meloydogine enterolobii* is not described as a major concern to cotton production, but a recent report of severe damage in a cotton cultivar resistant to *M. incognita* warned about the epidemic importance of this species. The genetic resistance is a promising approach to manage root-knot nematodes (RKN). This study aims to identify sources of resistance to *M. enterolobii* in Embrapa's cotton germplasm, which exhibit resistance to *M. incognita* and/or *Rotylenchulus reniformis*. In this study, 24 accessions including *Gossypium hirsutum* (Upland cotton), *G. barbadense* (Pima cotton), interspecific hybrids, and *G. arboreum* were examined for their susceptibility or resistance to *M. enterolobii*. The experiment involved six plants of each genotype under greenhouse conditions and was repeated at two different times. The inoculations were made with 10000 *M. enterolobii* eggs and after a period similar to field conditions (120 days), the following variables were evaluated: gall index, egg mass index, total number of eggs/gram of root and the reproduction factor (RF). The susceptible control, 'FM 966', displayed high reproduction factor (RF: 86.88 - 102.20) in both experiments, indicating the aggressiveness of the *M. enterolobii* population. Upland genotypes and Upland x Pima hybrids exhibited resistance levels ranging from moderately (MR) to resistant (R) and all *G. barbadense* genotypes were classified as susceptible, except 'Tanguis' and 'Pima California,' which were MR. *Gossypium arboreum* genotype was considered susceptible in both experiments. Genotype CNPA GO 2002-2043/5 showed consistently resistance

(reduction > 90%). Despite the aggressiveness of *M. enterolobii,* some cotton genotypes used in our experiments with RKN resistance QTLs (*qMi-C11* and *qMi-C14*) demonstrated a significant residual effect (about 80%) in reducing this nematode population after 120 days of inoculation. Our study highlights the potential of selecting cotton genotypes resistant to *M. incognita*, *R. reniformis* and *M. enterolobii* as a viable strategy to mitigate the impact of nematodes on cotton crops.

Keywords: Cotton race, *Gossypium* spp., guava nematode, resistance genes, virulence.

Título. RESISTÊNCIA PARCIAL DO ALGODÃO A *Meloidogyne enterolobii*: EFEITO RESIDUAL DE QTLS CONHECIDOS E UMA NOVA FONTE DE RESISTÊNCIA NA REDUÇÃO DA REPRODUÇÃO DO NEMATOIDE.

Resumo - *Meloydogine enterolobii* não é descrito como um patógeno importante para a produção de algodão, no entanto, relatos recentes de danos severos em uma cultivar de algodão resistente a *M. incognita* alertou sobre a importância epidêmica dessa espécie. A resistência genética é uma abordagem promissora para o manejo de nematoides das galhas. Este estudo tem como objetivo identificar fontes de resistência a *M. enterolobii* no germoplasma de algodão da Embrapa, que apresentam resistência a *M. incognita* e/ou *Rotylenchulus reniformis*. Neste estudo, 24 acessos, incluindo *Gossypium hirsutum*, *G. barbadense*, híbridos interespecíficos e *G. arboreum*, foram avaliados quanto à suscetibilidade ou resistência a *M. enterolobii*. O experimento contou com seis plantas de cada genótipo em condições de casa de vegetação e foi repetido em dois tempos diferentes. As inoculações foram feitas com 10000 ovos de *M. enterolobii* e, após um período semelhante às condições de campo (120 dias), as seguintes variáveis foram avaliadas: índice de galhas, índice de massa de ovos, número total de ovos/grama de raiz

e o fator de reprodução (RF). O controle suscetível, 'FM 966', apresentou alto fator de reprodução (RF: 86,88 - 102,20) em ambos os experimentos, indicando a agressividade da população de *M. enterolobii*. Os genótipos de *G. hirsutum* e os híbridos entre *G. hirsutum* e *G. barbadense* apresentaram níveis de resistência variando de moderadamente (MR) a resistente (R). Os genótipos de *G. barbadense* foram classificados como suscetíveis, exceto 'Tanguis' e 'Pima California', que foram MR. O genótipo de *G. arboreum* foi considerado suscetível em ambos os experimentos. O genótipo CNPA GO 2002-2043/5 mostrou resistência consistente (redução > 90%). Apesar da agressividade de *M. enterolobii*, alguns genótipos de algodão usados nos experimentos com QTLs de resistência a *M. incognita* (*qMi-C11* e *qMi-C14*) demonstraram um efeito residual significativo (cerca de 80%) na redução da população de nematoides após 120 dias de inoculação. O estudo destaca o potencial de seleção genótipos de genótipos de algodão resistentes a *M. incognita*, *R. reniformis* e *M. enterolobii* como uma estratégia viável para mitigar o impacto dos nematoides nas lavouras de algodão.

Palavras chave: Genes de resistência, *Gossypium* spp., nematoide da goiabeira, raça algodão, virulência

1. Introduction

The genus *Gossypium* L. (collectively termed cotton) comprises more than 50 species dispersed globally. Independently, four species were domesticated - two allopolyploids from the Americas, *G. hirsutum* L. and *G. barbadense* L., and two diploids from Africa-Asia, *G. arboreum* L. and *G. herbaceum* L. (Wendel and Grover, 2015). *Gossypium hirsutum* L. *latifolium* Hutch, commonly known as Upland cotton, is an important commodity in the world´s agricultural economy and accounts for over 90% of cotton fiber production. The extra-long staple, *G. barbadense*, represents approximately 5% of world fiber production (Adams, 2015). Cotton crop is especially important for

Brazilian trade balance, the second world's largest exporter of this commodity and fourth in production (USDA, 2023). However, biotic agents such as nematodes can cause damage to this crop, considerably reducing its potential yielding and fibre quality (Robinson, 2008; Davis *et al*., 2014). So far, only three root-knot nematode (RKN) species of the genus *Meloidogyne* Göldi, 1887 have cotton parasite status: *M. incognita* (Kofoid and White, 1919) Chitwood, 1949 (races 3 and 4), which is considered the most important cotton nematode due to its widespread occurrence in crop areas and its potential damage (Starr *et al*., 2005); *M. acronea* Coetzee, 1956 isolated in southern Africa; and, more recently, *M. enterolobii* Yang and Eisenback, 1983 (= *M. mayaguensis* Rammah & Hirschmann, 1988), which was detected on cotton in the USA (Ye *et al*., 2013) and in Brazil (Galbieri *et al*., 2020; Souza *et al*., 2022).

Meloidogyne enterolobii, is a nematode species predominantly found on guava across multiple Brazilian states (Carneiro *et al*., 2021). Although it has been reported in other crops, its occurrence is relatively limited but can result in significant damage (summarized in Castagnone-Sereno, 2012; Carneiro *et al*., 2021). This nematode exhibits a wide host range, parasitizing over 67 cultivated plant species across 27 botanical families (Castillo and Castagnone-Sereno, 2020).

Historically, research indicated that the guava *M. enterolobii* population did not infect cotton (Carneiro *et al*., 2001, 2006). However, recent reports have emerged, revealing a new race of *M. enterolobii* capable of parasitizing cotton in Brazil, specifically targeting the resistant cotton variety 'IMA 5801 B2RF' in Minas Gerais and Bahia states (Galbieri *et al*., 2020; Souza *et al*., 2022). It is important to note that the occurrence in Bahia was confined to a specific area. In the United States, *M. enterolobii* has been identified in North Carolina's Goldsboro region, where it has inflicted substantial damage to soybean and cotton crops, as well as in resistant sweet potatoes in South Carolina (Ye

et al., 2013; Rutter *et al*., 2021). Consequently, it is now considered a quarantine pathogen in these affected regions since its initial detection (Ye *et al*., 2013).

Compared with other RKN species, *M. enterolobii* displays virulence against the most sources of root-knot nematode-resistance genes, which constitutes a challenge for its control. An example of its virulence, *M. enterolobii* develops on crop genotypes carrying resistance to the major species of *Meloidogyne*, including resistant cotton (*qMi-c11* and *qMi-c14* loci), sweet potato, tomatoes (*Mi-1*gene), potato (*Mh* gene), soybean (*Mir1* gene), bell pepper (*N* gene), sweet pepper (*Tabasco* gene), cowpea (*Rk* gene) and *Capsicum* rootstock 'Snooker' (*Me1* and *Me3*/*Me7* genes) (Castagnone-Sereno, 2012; Pinheiro *et al.*, 2015; Souza *et al.*, 2022). So far, only a few potential sources of resistance to *M. enterolobii* have been identified, such as the *Ma* gene in *Prunus cerasifera* Ehrh., or in some guava accessions (*Psidium* spp.), bell pepper (*Capsicum chinense* L.), and sweet potato (*Ipomoea batatas* (L.) Lam.) accessions (Claverie *et al*., 2011; Gonçalves *et al.*, 2014; Freitas *et al.*, 2014; Carneiro *et al.*, 2021; Rutter *et al.*, 2021). Recently, the guava rootstock 'BRS Guaraçá' was released in Brazil by Embrapa (Souza *et al*., 2018). This rootstock shows a high resistance to different *M. enterolobii* populations, including the cotton race (Souza *et al.*, 2024).

Genetic resistance is the most desirable strategy of plant disease management, mainly for soil-borne diseases as plant parasitic nematodes. Currently, there are two genetically mapped sources of resistance against *M. incognita* available for cotton: one from *Gossypium hirsutum* (Upland cotton) and another from *G. barbadense* (Pima cotton) (Gomez *et al.*, 2016; Lopes *et al.*, 2020). However, only resistance from *G. hirsutum,* originated from Auburn 623 RNR breeding line (Shepherd, 1974a), have been incorporated into commercial cultivars and confers a high level of resistance. In Brazil, many cotton cultivars have been recently developed and released with this resistance,

such as IMA 5801 B2RF, FM 912 GLTP, FM 970 GLTP, BRS 500 B2RF and BRS 800 B3RF. These cultivars have also been developed using transgenic technologies, incorporating resistance to Lepidopterans pests and herbicides (Belot *et al.*, 2020; Suassuna *et al.*, 2021). In the United States, several cotton cultivars with resistance genes to *M. incognita* are available from four different seed companies with these same genes from Upland cotton (Wheeler *et al*., 2020).

The near immune resistance in Auburn 623 RNR and its descendants is conferred by two QTLs and is originated from a transgressive segregation derived from the crossing between two moderately resistant accessions, Clevewilt 6–3–5 and Wild Mexican Jack Jones (WMJJ). The high resistance found in Auburn 623 RNR has been transferred through successive backcrosses to give rise resistant cultivars and breeding lines (Shepherd *et al.*, 1996). These lines have been used by different cotton breeding programs, mainly M-120, M-240 and M-315. Genetic resistance in M-lines appears to be determined by at least two QTLs (McPherson *et al.*, 2004) mapped on chromosomes 11 and 14 (Shen *et al.*, 2006; Ynturi *et al.*, 2006). The QTL *qMi-C11*, originating from Clevewilt 6–3–5, has a dominant gene effect on the gall formation. QTL *qMi-C14*, originating from WMJJ, has partial dominant gene effect and is associated with reduced egg production (Gutiérrez *et al.*, 2010; Shen *et al.*, 2010; Jenkins *et al.*, 2012). QTL *qMi-C11* is mapped in the interval of the SSR markers CIR069-CIR316 on chromosome 11, and QTL *qMi-C14* is in the interval between BNL3545 and BNL3661 on chromosome 14 (Shen *et al.*, 2010; Da Silva *et al.*, 2019). An epistatic interaction between the two genes confers near-immunity resistance to RKN in the genotypes carrying both QTLs (He *et al*., 2014).

Several studies have shown a high resistance of the M-315 RNR line or other cotton genotype having *qMi-C11* and *qMi-C14* (Shepherd *et al.*, 1996; McPherson *et al*.,

2004; Lopes *et al*., 2020;) and no virulent populations of *M. incognita* have been found against genotypes carrying the *qMi-C11* and *qMi-C14* QTLs (Souza *et al.*, 2022). However, some results suggested that these QTLs are not effective against *M. enterolobii* and the African cotton-root nematode, *M*. *acronea* (Page and Bridge, 1994; Galbieri *et al.*, 2020; Gaudin *et al.*, 2023).

Gossypium barbadense genotype CIR 1348 was identified as a new source of high level of resistance to *M. incognita* (Mota *et al*., 2013; Silva *et al*., 2014). In accession CIR1348 two QTLs were identified, one on chromosome 11 and another on chromosome 15, which are responsible for the high level of resistance to the nematode (Silva *et al*., 2014; Gomez *et al*., 2016). However, these loci did not were definitely identified and more studies are necessary. Additionally, the interspecific crossing of Upland and Pima cotton is limited due mainly to genetic barriers between the two species in the forms of divergent gene regulatory systems, accumulated gene mutations, gene order rearrangements and cryptic chromosomal structure, differences that have resulted in hybrid breakdown, hybrid sterility and selective elimination of genes (Zhang *et al*., 2014). These facts impose difficulties to include *G. bardadense* sources in breeding programs. Others cotton sources have been tested for resistance to *M. incognita*, for example, Mota *et al*. (2013) found an accession of *G. arboreum* with high resistance to *M. incognita*. In contrast to Upland cotton, *G. barbadense* and *G. arboreum* never were tested against *M. enterolobii* before.

The epidemiological implications of *M. enterolobii* in cotton is uncertain, although several authors have found populations of *M. enterolobii* pathogenic on this crop (Brito *et al.*, 2004; Ye *et al*., 2013; Galbieri *et al*., 2020; Souza *et al*., 2022). Thus, the pathogen is increasingly associated with natural infections in the crop, with reports in North Carolina, USA (Ye *et al*., 2013), and in Brazil (Galbieri *et al*., 2020; Souza *et al*.,

2022), infecting cotton genotypes that exhibit high resistance level to *M. incognita*. Other sources of resistance to *M. incognita*, such as *G. barbadense* and *G. arboreum* and new sources in *G. hirsutum*, have not yet been studied for resistance to *M. enterolobii*. The aim of this study was to identify new sources of resistance to *M. enterolobii* in cotton accessions, which exhibit resistance to *M. incognita* and/or *Rotylenchulus reniformis* Linford & Oliveira, 1940 and evaluate their reaction to *M. enterolobii* (cotton race), 120 days after nematode inoculation, close to the cotton *cutout*, the end of the boll loading period in the field conditions.

2. Materials and Methods

Twenty-four *G. hirsutum*, *G. barbadense* and *Gossypium arboreum* accessions used in this study came from the Embrapa germplasm collections and are detailed in Table 7. Some of these genotypes have been previously tested for *M. incognita* and *R. reniformis* resistance, and include modern or obsolete cultivars, breeding lines and wild accessions. *Gossypium hirsutum* cv. FiberMax 966 (FM 966) was used as a susceptible check.

A *M. enterolobii* population collected in Paracatu (State of Minas Gerais, Brazil) was used in this study due to its pathogenicity on cotton (Galbieri *et al.*, 2020). Identification of the species was done using the Esterase (Est) phenotype (Carneiro and Almeida, 2001) and SCAR markers (Tigano *et al*., 2010). Prior to inoculation, the population was multiplied on tomato (*Solanum lycopersicum* cv. Santa Clara) for 3 or 4 months under greenhouse conditions. Eggs were extracted from infected roots according to the modified method described by Hussey and Barker (1973), using 0.5% NaOCl and a blender for 30 s, instead of manual agitation.

Table 7 - Cotton genotypes evaluated for reaction to *Meloidogyne enterolobii* cotton race.

Genotype	Species	Remark
02-139 / 2	*Gossypium hirsutum* x *G. barbadense*	Resistance to *Mi*[1] (alleles CIR 316 and BNL 3661) and partial resistance to *Rr*[2]
02-34 / 31	*G. hirsutum* x *G. barbadense*	Resistance to *Mi* (alleles CIR 316 and BNL 3661) and partial resistance to *Rr*
02-78 / 28	*G. hirsutum* x *G. barbadense*	Resistance to *Mi* (alleles CIR 316 and BNL 3661) and partial resistance to *Rr*
19-2056	*G. hirsutum*	Partial resistance to *Mi* (allele CIR 316)
19-2398	*G. hirsutum*	Complete resistance to *Mi* (alleles CIR 316 and BNL 3661)
Wild Mexico Jack Jones	*G. hirsutum*	Partial resistance to *Mi* (allele BNL 3661)
CNPA GO 2002-2043/5	*G. hirsutum*	Partial resistance to *Mi* (without known R alleles)
CNPA GO 2017-1026 B2RF	*G. hirsutum*	Complete resistance to *Mi* (alleles CIR 316 and BNL 3661)
FM 966	*G. hirsutum*	*Mi* susceptibility control
IMA 5801 B2RF	*G. hirsutum*	Complete resistance to *Mi* (alleles CIR 316 and BNL 3661)
Mocó Currais	*G. hirsutum* race Marie Galant	Wild population from semi-arid zone
M 315	*G. hirsutum*	Complete resistance to *Mi* (alleles CIR 316 and BNL 3661)
Clevewilt 6	*G. hirsutum*	Partial resistance to *Mi* (linked to allele CIR 316)
LA 887	*G. hirsutum*	Partial resistance to *Mi* (descendant of Clevewilt 6)
T3-6 (CIR 1348 x FM 966)	*G. barbadense* x *G. hirsutum*	Partial resistance to *Mi* (without known R alleles)
CIR 1548	*G. arboreum*	Without known R alleles to *Mi*
Karnak	*G. barbadense*	Pima cotton with no information of resistance to *Mi*
Menoufi	*G. barbadense*	Pima cotton with no information of resistance to *Mi*
Pima California	*G. barbadense*	Pima cotton with no information of resistance to *Mi*
Tanguis	*G. barbadense*	Pima cotton with no information of resistance to *Mi* (PI 316400)
CNPA H7	*G. barbadense*	Pima cotton with no information of resistance to *Mi*
Sakha 3	*G. barbadense*	Pima cotton with no information of resistance to *Mi*
CIR 1348	*G. barbadense*	Partial resistance to *Mi*
IAC 24	*G. hirsutum*	Partial resistance to *Mi* (without known R alleles)
IAC 25 RMD	*G. hirsutum*	Partial resistance to *Mi* (allele CIR 316)

[1]*Mi*: *Meloidogyne incognita*; [2]*Rr*: *Rotylenchulus renirformis*

Six plants of each genotype arranged in a randomized block design were grown in pots 40 cm high and 20 cm diameter filled with a mixture (1:1) of autoclaved soil and Plantmax® compost, in a greenhouse maintained at 25 – 30°C. Twenty-one days after seed emergence, pots were inoculated with 10000 *M. enterolobii* cotton race eggs by pipetting the nematode suspension in holes around the stem base. The experiment was

repeated at two different times to confirm the results of the previous test. The cotton plants were watered and fertilized as necessary.

Experiment 1 took place from March to August 2022 (Autumn-Winter), while Experiment 2 occurred from December 2022 to May 2023 (Summer-Autumn). One hundred and twenty days after inoculation (dai), plants were uprooted, the root system rinsed under tap water, and the roots weighed. Roots were stained with phloxin B (15 mg/ L) and evaluated for gall and egg mass numbers (galling index, GI; egg mass index, EMI), using a scale where 0 = no galls or egg masses; 1 = 1–2 galls or egg masses; 2 = 3–10 galls or egg masses; 3 = 11–30 galls or egg masses; 4 = 31–100 galls or egg masses; and 5 > 100 galls or egg masses per root system (Hartman and Sasser, 1985).

Eggs were extracted using the modified method described by Hussey & Barker (1973), using 1.0% NaOCl and a blender for 30 seconds, and the total egg number per plant was calculated. The reproduction factor (RF) was calculated as RF = FP/IP, where FP = final nematode population and IP = initial nematode population (IP = 10000) (Oostenbrink, 1966). Due the non-normal distribution of the RF values, this parameter was transformed to $\sqrt{x+1}$ and, after obtained normality the analysis of variance was performed, and the averages were compared using Scott–Knot's test at the 5% probability level. Pearson's correlation analysis was used for each variable. All analyses were performed in the R software (R Core Team, 2022).

The reactions of cotton genotypes to *M. enterolobii* were classified according to the reproduction criteria (RF) and percentage of population reduction (% PR). This criterion established host susceptibility/resistance was defined as follows: nematode reproduction similar or superior to the control ('FM966') designates a

susceptible host (S), a moderately resistant plant (MR) presented intermediary RF and a resistant plant (R) low RF, based on statistical analyses.

3. Results

The susceptible control, 'FM 966' (lacking resistance genes), displayed significant values of reproduction factors (RF: 102.20 and 86.88) in both experiments, highlighting the high aggressiveness of the *M. enterolobii* population used in this study (Tables 8 and 9). Symptoms of large galls in cotton roots were observed for the standard and other susceptible genotypes (Fig. 12a, b) and milder symptoms in genotypes considered resistant (Fig. 12 c, d). Our results showed that the genotype CNPA GO 2002-2043/5 was consistently the most resistant (R) to *M. enterolobii* in both experiments, with RFs 9 in the first experiment and 4.38 in the second (Tables 8 and 9), resulting in a population reduction of more than 90% compared with susceptible control. Other genotypes (Tables 8 and 9) submitted to similar statistical analysis (populational reduction from 74.2 to 89.7%) were also considered resistant (LA 887, 02-139/2, T3-6, Wild Mexico Jack Jones, BRS 800 B3RF, Clevewilt 6, CNPA GO 2017-1026 B2RF, IMA 5801 B2RF, 02-78/28, M 315, 02-34/31 and 19-2056).

Table 8 - Experiment 1: Reaction of cotton accessions inoculated with 10000 eggs of *Meloidogyne enterolobii* (cotton race), based on the variables fresh root weight (FRW), gall index (GI), egg-mass index (EMI), number of eggs per gram of roots (NEGR), reproduction factors (RF) and percentage of population reduction (% PR) of the cultivars evaluated at 120 DAI. March to August 2022 (Autumn-Winter).

Genotype	FRW	GI	EMI	NEGR	RF	%PR[3]	Phenotype[4]
NPA GO 2002-2043/5	30.3 a[1]	5.0	4.8	3112.9 a	9.00 a	91.2	R
LA 887	62.8 c	4.8	4.3	1963.1 a	10.54 a	89.7	R
02-139/2	22.8 a	4.5	4.0	4721.1 b	10.57 a	89.7	R
T3-6 (CIR 1348 x FM 966)	78.1 c	5.0	5.0	1589.3 a	11.00 a	89.2	R
Wild Mexico Jack Jones	124.0 d	4.5	4.3	962.9 a	11.81 a	88.4	R

19-2398	22.5	a	5.0	4.8	5438.8	b	12.38	a	87.9	R
Clevewilt 6	48.9	b	5.0	4.0	3170.8	a	13.87	a	84.4	R
BRS 600 B3RF	17.4	a	4.5	4.5	7848.4	b	14.12	a	86.2	R
CNPA GO 2017-1026 B2RF	80.3	d	5.0	5.0	1930.7	a	14.90	a	85.4	R
IMA 5801 B2RF	55.3	c	5.0	4.3	3811.8	a	16.88	a	83.8	R
02-78/28	18.7	a	4.3	4.0	7062.2	b	16.95	a	83.4	R
M 315	98.3	d	5.0	5.0	1846.6	a	17.06	a	83.3	R
02-34/31	25.8	a	4.8	4.7	7112.0	b	18.36	a	82.0	R
19-2056	33.0	a	5.0	5.0	8307.1	b	20.92	a	79.9	R
IAC 24	86.0	d	5.0	5.0	4294.9	b	43.47	b	57.5	MR
Tanguis	55.8	c	5.0	5.0	7383.9	b	48.61	b	52.4	MR
Pima California	83.2	d	5.0	5.0	5604.1	b	51.52	b	50.7	MR
Karnak	92.8	d	5.0	5.0	5887.4	b	53.55	b	46.6	MR
CIR 1548	51.2	c	5.0	5.0	11150.8	c	66.73	c	34.7	S
IAC 25RMD	90.3	d	5.0	5.0	6491.9	b	69.16	c	32.3	S
CIR 1348	96.0	d	5.0	5.0	6804.9	b	75.83	c	27.4	S
FM 966[5]	109.6	d	5.0	5.0	8001.9	b	102.20	d	0.0	S
Menoufi	62.3	c	5.0	5.0	15613.7	c	122.52	d	0.0	S
Sakha 3	66.6	c	5.0	5.0	29140.3	d	162.62	e	0.0	S
Mocó Currais	105.3	d	5.0	5.0	14099.6	c	175.56	e	0.0	S
CV (%)[2]	16.36		-	-	30.25		21.07		-	-

[1]Means followed by the same lowercase letter in the column do not differ statistically from each other according to the Scott-Knott test at 5% probability ($P \leq 0.05$). The mean values were transformed to $\sqrt{x + 1}$ and the original data are presented in the table. [2]CV = Coefficient of variation after transformation. [3] % PR: Percentage of Population Reduction using the control FM 966 as reference. [4]Classification criteria using statistical analyses, where S = Susceptible, MR = Moderately resistant and R = Resistant. [5] Cotton cultivar ('FM 966') used as a standard control for the classification.

Table 9 - Experiment 2: Reaction of cotton accessions inoculated with 10000 eggs of *Meloidogyne enterolobii* (cotton race), based on the variables fresh root weight (FRW), gall index (GI), egg-mass index (EMI), number of eggs per gram of roots (NEGR), reproduction factors (RF) and percentage of population reduction (% PR) of the cultivars evaluated at 120 DAI. December 2022 to May 2023 (Summer-Autumn).

Genotype	**FRW**		**GI**	**EMI**	**NEGR**		**RF**		**%PR[3]**	**Phenotype[4]**
CNPA GO 2002-2043/5	10.8	a[1]	4.0	4.0	3887.5	b	4.38	a	95.0	R
M 315	86.8	e	5.0	5.0	1327.4	a	11.73	a	86.5	R
Wild Mexico Jack Jones	109.1	f	4.8	4.8	1088.8	a	12.53	a	85.6	R
T3-6 (CIR 1348 x FM 966)	129.4	g	5.0	5.0	964.8	a	13.47	a	84.5	R
CNPA GO 2017-1026 B2RF	88.9	e	5.0	5.0	1583.0	a	14.40	a	83.4	R
IMA 5801 B2RF	43.4	c	4.3	4.2	3824.8	b	14.49	a	83.3	R

BRS 800 B3RF	27.5	b	4.5	4.2	5232.1	b	15.20	a	82.5	R
02-139/2	27.6	b	4.3	4.2	5187.8	b	17.47	a	79.9	R
Clevewilt 6	55.8	c	5.0	4.3	3002.8	a	17.69	a	79.6	R
LA 887	71.9	d	4.5	4.5	2371.0	a	17.87	a	79.4	R
02-78/28	28.6	b	4.5	4.2	5353.3	b	18.03	a	79.2	R
02-34/31	27.8	b	4.5	4.2	7192.2	b	20.57	a	76.3	R
19-2056	32.4	b	5.0	4.5	6763.9	b	22.39	a	74.2	R
IAC 24	82.4	e	5.0	5.0	3792.6	b	36.08	b	58.5	MR
Pima California	75.1	d	5.0	5.0	4572.2	b	41.12	b	52.9	MR
Karnak	58.1	c	5.0	5.0	6893.9	b	44.08	b	49.3	MR
Tanguis	49.7	c	5.0	5.0	8163.1	c	45.94	b	47.2	MR
CIR 1548	51.7	c	5.0	5.0	10229.2	c	61.99	c	28.6	S
CIR 1348	103.6	f	5.0	5.0	5825.0	b	71.27	c	18.0	S
FM 966[5]	93.8	e	5.0	5.0	7909.4	c	86.88	c	0	S
IAC 25RMD	92.3	e	5.0	5.0	8607.4	c	93.37	c	0	S
Menoufi	67.7	d	5.0	5.0	15242.4	c	121.61	d	0	HS
Sakha 3	47.8	c	5.0	5.0	31539.9	d	131.49	d	0	HS
Mocó Currais	144.3	g	4.7	4.5	10784.5	c	186.14	e	0	HS
CV(%)[2]	12.25		-	-	31.63		26.05		-	-

[1]Means followed by the same lowercase letter in the column do not differ statistically from each other according to the Scott-Knott test at 5% probability ($P \leq 0.05$). The mean values were transformed to $\sqrt{x + 1}$ and the original data are presented in the table. [2]CV = Coefficient of variation after transformation. [3] % PR: Percentage of Population Reduction using the control FM 966 as reference. [4]Classification criteria using statistical analyses, where S = Susceptible, MR = Moderately resistant and R = Resistant. [5]Cotton cultivar ('FM 966') used as a standard control for the classification.

Other four genotypes (IAC 24, Pima California, Karnak and Tanguis) showed intermediary population reduction (46.6 to 58.5 %) and were considered MR. This tendency was observed in both experiments (Tables 8 and 9). The other genotypes (CIR1548, CIR 1348, FM966, IAC 25RDM, Menoufi, Sakha 3, Mocó Currais) were considered susceptible (S) or highly susceptible (HS) according to statistical analyses, with no population reduction, or around 30% of reductions. Generally, genotypes demonstrating a population reduction, around 70-80% (R) are deemed to exhibit a highly promising level of resistance against *M. enterolobii* in cotton.

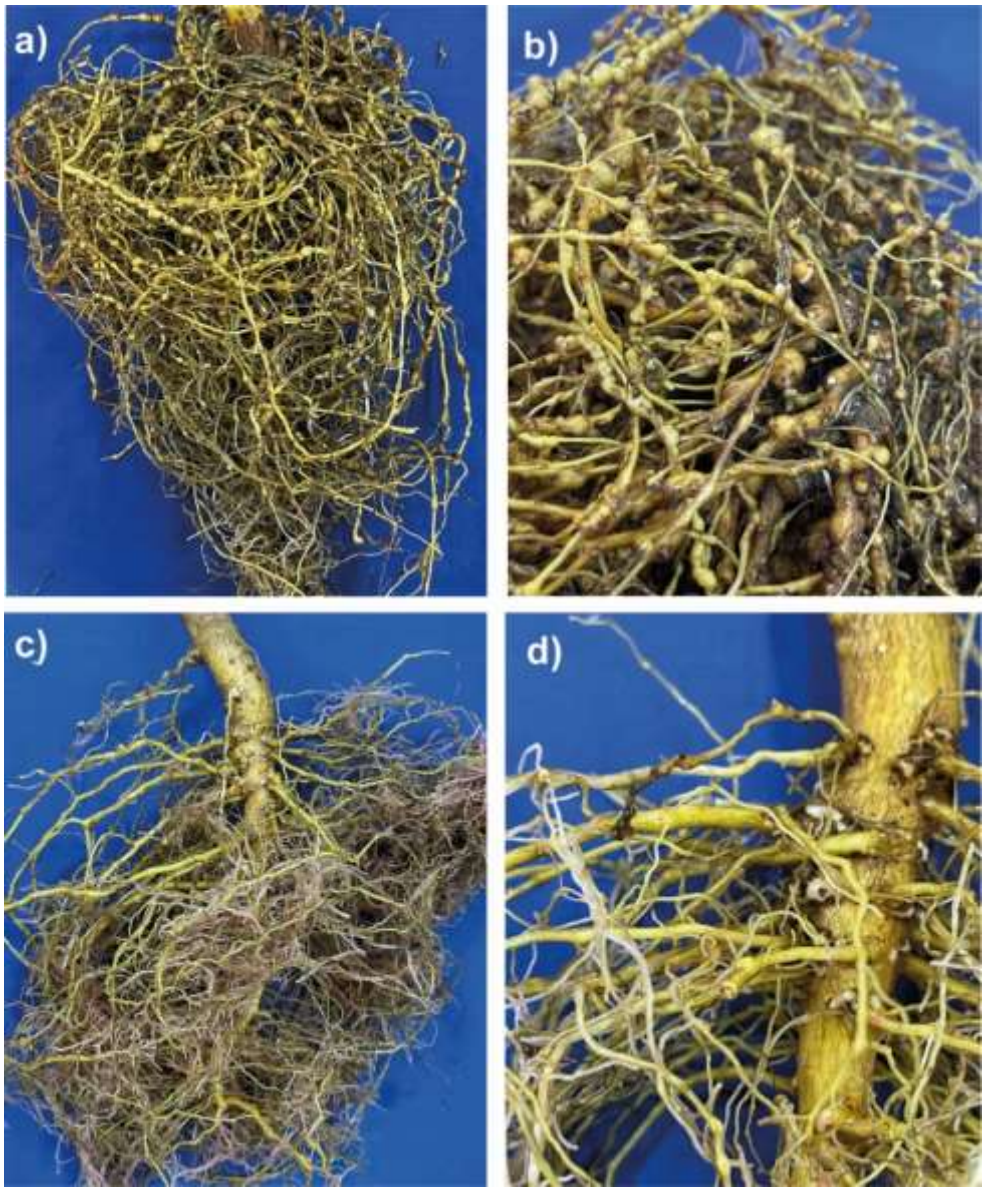

Figure 12 - Roots inoculated with 10000 eggs of *Meloidogyne enterolobii* cotton race. Root of susceptible check 'FM 966' (a), showing abundant and large galls (b); Root of genotype CNPA GO 2002-2043/5 (c) with few galls present (d).

In general, Upland genotypes (*G. hirsutum*) and Upland x Pima hybrids (*G. hirsutum* x *G. barbadense*) displayed resistance levels ranging from resistant (R) to moderate resistant (MR) in both experiments. Reproduction factors (RFs) varied from 9.00 to 53.55 in the first experiment and 4.38 to 45.94 in the second experiment. However, the Upland genotypes IAC 25RMD, FM 966 (susceptible control), and Mocó-Currais

were consistently classified as susceptible (S) or highly susceptible (HS) in both experiments (RF from 69.2 to 175.6) (Tables 8 and 9).

All *G. barbadense* genotypes were classified as susceptible (S) or highly susceptible (HS), with RFs ranging from 51.52 to 162.62 in the first experiment and 44.08 to 131.49 in the second. The exceptions were 'Tanguis' with an RF of 48.61 in the first experiment and 'Pima California' with an RF of 41.12 in the second experiment, both classified as moderately resistant (MR) (Tables 8 and 9). The only *G. arboreum* genotype, CIR 1548, was classified as susceptible (S), with RFs of 66.73 and 61.99 in the first and second experiments, respectively (Tables 8 and 9).

Throughout both evaluation periods (Experiment 1 and 2), the measurements of fresh root weight (FRW) indicated healthy growth for all cultivars, irrespective of nematode parasitism. There were a few exceptions, in ‘CNPA GO 2002-2043/5’, ‘02-139/2’, ‘19-2398’, ‘02-78/28’, ‘02-34/31’, and ‘19-2056’, ranging from 22.8 g to 33.0 g in the first experiment. In the second experiment, FRW was impacted in ‘02-34/31’ and ‘19-2056’ (10.8 and 18.3 g, respectively) (Tables 8 and 9).

Throughout both evaluation periods (Experiment 1 and 2), the measurements of fresh root weight (FRW) indicated good growth for all plants depending on the genetic characteristics of each genotype, irrespective of nematode parasitism (Tables 8 and 9).

The other variables evaluated may be considered of secondary importance, since were evaluated only symptoms and using indices without statistical analyses. The averages of gall index and egg-mass index (GI and EMI) in Experiment 1 (Table 8) ranged from 4.3 to 5.0 for both variables. In addition, in Experiment 2 ranged from 4.0 to 5.0 GI and EMI, respectively (Table 10). The lowest rates of galls of *M. enterolobii* were in the genotypes ‘02-78/28’ and ‘02-139/2’ in the first experiment (Table 8), while for the

second, the lowest indices were for ‘CNPA GO 2002-2043/5’ and ‘BRS 600 B3RF’; in addition, these genotypes allowed a low reproduction of the nematode (Table 9).

Table 10 - Pearson correlation coefficients (R) between the variables: root weight (RW), gall index (GI), egg mass index (EMI), number of eggs per root (ER), number of eggs (g root)$^{-1}$ (EGR) and reproduction factor (RF) in cotton plants infected by *Meloidogyne enterolobii* in two experiments.

Experiment 1							Experiment 2						
	RW	GI	EMI	ER	EGR	RF		RW	GI	EMI	ER	EGR	RF
RW	-	-	-	-	-	-	RW	-	-	-	-	-	-
GI	0.4**	-	-	-	-	-	GI	0.3**	-	-	-	-	-
EMI	0.4**	1.0**	-	-	-	-	EMI	0.3**	0.8**	-	-	-	-
ER	0.4**	0.4**	0.5**	-	-	-	ER	0.4**	0.3**	0.3**	-	-	-
EGR	-0.1*	0.5**	0.5**	0.4**	-	-	EGR	-0.1^{ns}	0.2*	0.2**	0.6**	-	-
RF	0.3**	0.4**	0.4**	0.9**	0.4**	-	RF	0.4**	0.3**	0.3**	1**	0.6**	-

Significance level: * P < 0.05, ** P < 0.01, NS = non-significant.

Summarizing in Experiment 1 (Table 8), the reproduction factors (RF) for 'Sakha 3' and 'Mocó-Currais' stood out with the highest RFs of 175.56 and 162.62, respectively. Following closely were 'Menoufi' and the susceptible control 'FM 966' (all lacking resistance genes). The genotypes CIR 1548 (*G. arboreum*), IAC 25RMD (*G. hirsutum*), and CIR 1348 (*G. barbadense*) formed another statistical group with RFs ranging from 53.55 to 75.83, all classified as susceptible (S). 'IAC 24,' 'Tanguis,' 'Pima California,' and 'Karnak' constituted the fourth statistical group with RFs between 43.97 and 53.55 and were considered MR. The remaining genotypes (CNPA GO 2002-2043/5, LA 887, 02-139/2, T3-6 (CIR 1348 x FM 966) RC2F2:4, Wild Mexico Jack Jones, BRS 800 B3RF, Clevewilt 6, CNPA GO 2017-1026 B2RF, IMA 5801 B2RF, 02-78/28, M 315, 02-34/31, and 19-2056) were grouped and classified as resistant (R), with RFs ranging from 9.0 to 20.92 (Table 8). In Experiment 2 (Table 9), the results were similar to Experiment 1, showing data consistency and repeatability.

In general, Pearson correlation analysis of the evaluation parameters revealed positive and significant correlations (Table 10). However, in the first experiment,

RW/EGR, RW/ER, and RW/RF exhibited low to moderately negative correlations; and RW/EGR displayed a low negative correlation in the second experiment. The reproduction factor (RF) parameter displayed strong associations with total eggs per root (ER) and eggs per gram of root (ERF) and showed slightly linked to other parameters such as gall and egg mass indices (GI and EMI). This suggests that GI and EMI may not be reliable variables for assessing *M. enterolobii* reproduction (RF) on cotton (Table 10).

4. Discussion

Meloidogyne enterolobii is an emerging pathogen on cotton crops and have been reported causing damage on cotton varieties carrying the two main resistance QTLs (*qMi-c11* and *qMi-c14*) for *M. incognita* (Galbieri *et al*., 2020; Souza *et al*., 2022). However, we hypothesize that cotton genotypes that are resistant to other nematodes species can also display some resistance level in reducing damage and reproduction of *M. enterolobii,* and consequently, contribute to managing these harmful nematodes in the agricultural systems.

In general, the pathogen isolate used in our study reproduced in similar levels in Experiment 1 and 2, with slight variations confirming the results obtained. In both experiments, the nematode isolate was able to reproduce on genotypes that contain the two mapped RKN resistance QTLs (*qMi-c11* and *qMi-c14*) and/or the reniform nematode (RN) resistance QTL, and induced root galling and variable RF values. These data support the virulence of *M. enterolobii* race 2 to cotton resistance genes previously reported by Souza *et al.* (2022) and Gaudin *et al.* (2023). Nevertheless, the breeding line CNPA GO 2002-2043/5 exhibited a population reduction of more than 90%, thus establishing it as resistant (Taylor, 1967; Hussey and Janssen, 2002). Various genotypes possessing resistance QTLs (*qMi-C11* and *qMi-C14*) for *M. incognita* displayed an approximate 80%

reduction and were similarly classified as resistant. This observation is particularly noteworthy as it reflects a significant population reduction over an extended period, similar to the cotton period to reach the physiological *cutout* in the field conditions, meaning that cotton plant is near to the end of the blooming period, and it has reached its capacity for supporting fruiting positions and likely will not development any additional fruiting sites resulting in harvestable bolls. Gaudin *et al*. (2023) evaluated five genotypes carrying resistance loci *qMi-C11* and *qMi-C14* for *M. incognita* and/or the Ren^{barb2} for *R. reniformis*. Their study, conducted over a six-week revealed low Reproduction Factors (RF) after this short period, suggesting that these resistance loci were ineffective against *M. enterolobii* populations collected in the USA. Their RFs were lower (about 21 times) than the RFs observed in our study. It is important to highlight that the low RFs observed in susceptible checks (Gaudin *et al.*, 2023) can compromise resistance assessments due to the lack of sufficient contrast between susceptible and potentially resistant plants. For semi-perennial or perennial crops such as cotton, guava, coffee, among others, evaluations after long periods of time with significant contrasts in RFs between control and plants with resistance genes are essential to confirm susceptibility/resistance or plant host status (Freitas *et al.*, 2014, 2017; Lopes *et al.*, 2019; Almeida *et al.*, 2023). In contrast to Gaudin *et al*. (2023), our longest experiment, lasting approximately 4 pathogen life cycles (120 days) featuring high levels of nematode reproduction provide a reliable assessment framework, revealing that genotypes carrying one or both resistance QTLs (*qMi-C11* and *qMi-C14*), such as '02-139/2', 'BRS 800 B3RF', 'CNPA GO 2017-1026 B2RF', 'IMA 5801 B2RF', '02-78/28', 'M-315', '02-34/31', 'LA 887', 'T 3-6', 'Wild Mexico Jack Jones', 'Clevewilt 6', and '19-2056' exhibited a significant reduction in reproductive rates compared to the susceptible control 'FM 966'.

The residual effect of resistance genes was demonstrated by Bost and Triantaphyllou (1982) in a classical work with segregant tomato *Mi* lines, that exhibited a residual effect in virulent populations of *M. incognita.* In other example, Gabriel *et al*. (2022) demonstrated that the resistant tomato rootstock 'Muralha' (*Mi Mi1.2*) reduced the reproduction of a virulent population of *M. javanica* (Treub, 1885) Chitwood, 1949 in 60 % when compared to the susceptible cultivar Santa Clara. However, this effect had not been previously reported in cotton.

The genotype CNPA GO 2002-2043/5 was considered resistant to *M. enterolobii* in both experiments, according to statistical analyses due the low levels of nematode reproduction compared to the susceptible control. This genotype was originated from a tri-parental cross of cotton cultivars BRS Aroeira, U206 and Delta Opal [BRS Aroeira x (U206 x Delta Opal)]. The cultivar BRS Aroeira was released in 2001 for use in low-cost production system; this cultivar is resistant to multiple diseases, including cotton ramulosis, cotton blue disease, *Stemphylum* leaf-blight, and tolerance to bacterial infections, ramularia leafspot and RKN and fusarium wilt disease complex (Freire *et al*., 2009). BRS Aroeira is derived from a broad-based population, involving diverse parents, including Acala types, Brazilian landraces, Sealand and *G. hirsutum* race Marie Galant. This diverse genetic background can explain the partial resistance to *M. enterolobii* found in CNPA GO 2002-2043/5. It may be regulated by minor genes with additive effect (quantitative resistance) against other phytopathogenic organisms may lead to resistance against *M. enterolobii* (Barbary *et al.*, 2016), in contrast to single or pair of major resistance genes (R-genes) common in Upland cotton resistance (*qMi-C11* and *qMi-C14* QTLs) (Wheeler *et al.*, 2020). Further experiments will be needed to prove the additive effect of partial resistance of CNPA GO 2002-2043/5.

Few sources of resistance to *M. enterolobii* have been identified, such as the *Ma* gene in *P. cerasifera*, guava accessions, pepper, sweet potato and the recently released guava rootstock 'BRS Guaraçá' (Claverie *et al.*, 2011; Costa *et al*., 2012; Freitas *et al.*, 2014; Gonçalves *et al.*, 2014; Carneiro *et al*., 2021; Rutter *et al.*, 2021; Souza *et al.*, 2024). Emphasizing that 'BRS Guaraçá' follows a dominant resistance model, controlled by two genes with epistatic effects, where the presence of only one dominant allele conditions the hybrid's resistance to *M. enterolobii* (Santos *et al*., 2022). In all these afore mentioned cases, the resistance against the guava nematode is conferred by dominant genes (qualitative resistance), according to the low RF values (RF < 1) and the suppression of symptoms reported in these studies, differing from the possible quantitative resistance observed on cotton genotypes in our study.

Meloidogyne acronea has been confirmed as a potentially serious pathogen of cotton, displaying virulence to *M. incognita*-resistant American cultivars, like Auburn 623 and Clevewilt. *Meloidogyne acronea* is regarded as indigenous to semi-arid regions of southern Africa and its occurrence within the natural habitat for *G. herbaceum* var. *africanum* suggests that there may have been co-evolution between these two species, enhance its ability to parasite cotton and overcome their resistance mechanism (Page and Bridge, 1994). This is not the case of *M. enterolobii*, which was found causing damage to cotton just recently in Brazil and USA (Ye *et al*., 2013; Galbieri *et al*., 2020; Souza *et al*., 2022). Although present in Africa (Santos *et al*., 2018b), *M. enterolobii* has never been observed infecting cotton (Onkendi *et al*., 2014; Santos *et al*., 2018b; Castillo and Castagnone-Sereno, 2020), emerging the question on the geographic origin of the cotton race and its relationship with *Gossypium* spp.

Gossypium arboreum possesses several favourable traits for cotton production that are lacking in the Upland cotton cultivars. These include drought tolerance, resistance to

diseases such as root rot, and pests as bollworms and aphids (Mehetre *et al.*, 2003), as well as high resistance to *M. incognita* (Mota *et al.*, 2013). However, our results indicate that the genes present in this species are ineffective against *M. enterolobii*, as evidenced by the high RF values (61.99 - 66.73) observed in 'CIR 1548', consistently classified as susceptible in both experiments.

To achieve optimal resistance through breeding, it is crucial to select progeny carrying combinations of genes that are homozygous for resistance. Interestingly, even parents displaying moderate to high susceptibility can contribute to nematode resistance through transgressive segregation (Wang *et al*., 2008). These crosses can yield highly resistant lines, even when both parents exhibit susceptibility; a notable example of this is the backcrossing of two moderately resistant accessions, Clevewilt-6 and Wild Mexican Jack Jones (Shepherd, 1974a), resulted in the highest level of resistance to RKN known to date in cotton. Such transgressive segregants can serve as valuable sources of improved resistance in crop breeding (Wang *et al*., 2008; Ulloa *et al*., 2011).

In this study, all *G. hirsutum* accessions with one or two QTL for *M. incognita* and *R. reniformis* were classified as resistant (R) compared to the susceptible check 'FM 966, even in the case of 'CNPA GO 2002-2043/5' which does not have any resistance QTL mapped. This suggests the existence of a genetic background influencing the phenotype, reducing nematode reproduction. However, it is noteworthy that it does not completely prevent nematode reproduction to the same extent as observed for *M. incognita*. Yet, there is limited literature available on the influence of plant genetic background on the expression of resistance to nematodes, such as tomato, potato, pepper and soybean (Jacquet *et al*., 2005; Djian-Caporalino *et al*., 2007; Verssiani *et al*., 2023).

A low correlation was identified in GI/RF and EMI/RF comparisons, indicating that when performing screening in the field, the visual assessment of the galls and egg

masses cannot be directly linked to the nematode reproduction, being the greenhouse screening and the nematode reproduction assessment more suitable for evaluation of cotton resistance (Hussey and Janssen, 2002). This low correlation aligns with the findings of Lopes *et al*. (2019) who also observed low correlation between GI and EMI with RFs in cotton inoculated with *M. incognita* race 3.

In conclusion, our study emphasizes the potential of selecting cotton genotypes resistant to *M. incognita* and/or *R. reniformis* as an effective strategy to mitigate the impact of *M. enterolobii* on cotton crops. Despite the virulence of the *M. enterolobii* isolate used in our experiments, known resistance QTLs (*qMi-C11* and *qMi-C14*) demonstrated a significant residual effect in reducing nematode reproduction. On the other hand, the cotton-breeding line CNPA GO 2002-2043/5, with no previously described nematode resistance QTL, exhibited an interesting resistance level to *M. enterolobii.* As a breeding strategy, pyramiding all known QTLs for resistance to nematodes in a favourable genetic background with a partial level of resistance to *M. enterolobi*, as in CNPA GO 2002-2043/5, could result in a higher resistance response.

These findings challenge traditional classifications and highlight the need for a nuanced understanding of resistance mechanisms. Moreover, this study highlighted the need for phenotyping for longer periods, in the case of perennial and semi-perennial plants, in order to give the nematode enough time to fully manifest its development and reproduction. It also emphasized the importance of selecting highly susceptible controls that enable accurate comparison with the plants to be phenotyped (Taylor, 1967). Our results offer valuable insights for cotton breeding programs, emphasizing the importance of genetic backgrounds and the potential for transgressive segregation in developing nematode-resistant cultivars for sustainable cotton cultivation.

5. Conclusions

In conclusion, our study underscores the critical role of selecting cotton genotypes resistant to *M. incognita* and/or *R. reniformis* as a valuable strategy to reduce the effects of *M. enterolobii* on cotton cultivation. Despite the high virulence exhibited by the *M. enterolobii* isolate utilized in our experiments, the presence of known QTL's (*qMi-C11* and *qMi-C14*) shown a significant residual effect, markedly reducing nematode reproduction. Conversely, the cotton breeding line CNPA GO 2002-2043/5, lacking previously identified nematode resistance genes, demonstrated remarkable resistance, highlighting the existence of alternative genetic factors influencing resistance phenotype on cotton genotypes. This study emphasizes the necessity of long-term evaluations time to accurately estimate resistance levels against *M. enterolobii*, ensuring sustainable cotton production in the face of evolving nematode challenges.

General Conclusions

• Our study identified *M. enterolobii* naturally infecting resistant cotton cultivar in Bahia state and high populations of *M. incognita* in areas indicated as resistant cotton by biochemical and molecular approaches (esterase phenotypes and SCAR markers). Greenhouse assays confirming the pathogenicity of *M. enterolobii* on cotton and its virulence in resistant cultivars. In contrast, *M. incognita* populations collected from western Bahia did not exhibit virulence to resistant cotton cultivars in the greenhouse assays, due probably incorrect field identification and/or seed mixture in on-farm seed production.

• Using the North Carolina Differential Host Test (NCDHT) to study 7 populations of *M. enterolobii* from Brazil, we can differentiate three populations classified as race 1 (do not

infecting cotton), and two populations were classified as race 2 based on their ability to parasitizes cotton plants. Comparison between the original cultivars recommended in the NCDHT and current cultivars suggested in our study showed few variations but did not invalidate the differential host test.

• RAPD and AFLP molecular markers were used to assess genetic variability. Overall, there was low global polymorphism, with 12.5% of amplified fragments being polymorphic. Polymorphism levels varied among populations, with higher variability observed between populations from different hosts. Sequences from different gene regions (*ITS, D2-D3, COXII*, and *HSP90*) were analyzed for phylogenetic relationships. Phylogenetic reconstructions did not show clustering of sequences based on host or race, except for slight grouping in *COXII*.

• Genotype CNPA GO 2002-2043/5 consistently showed the highest resistance to *M. enterolobii* in both experiments, with RFs resulting in a population reduction of over 90% compared to the susceptible control. Upland cotton genotypes and Upland x Pima hybrids generally displayed resistance levels ranging from resistant to moderately resistant in both experiments. All *G. barbadense* genotypes were classified as susceptible or highly susceptible, except for 'Tanguis' and 'Pima California,' which were moderately resistant. The only *G. arboreum* genotype, CIR 1548, was classified as susceptible. Pearson correlation analysis revealed that specific correlations, particularly involving root gall or egg mass index, exhibited low to moderately negative associations with reproduction factor.

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